University of Alberta

Impaired HDL Formation in Niemann-Pick type C Disease and Epithelioid Arterial Smooth Muscle Cells

by

Hong Yeob Choi ©

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DEDICATION

To my wife, Jung Hwa Kang

As I think back over the years that we have passed for this study, I see a series of pictures of you. Some pictures are bright and some are dark, but I see your heart with full of support and love from all the pictures. Although it is not enough, I just want to say "thank you" for giving me strength and hope when I afraid, and for sharing the joys and sorrows of life with me.

Thank you also for bringing us our wonderful children, Shiwon and Senna. Their smile has a miracle power to cheer me up. They make my life more meaningful and lovely.

ABSTRACT

ATP-binding cassette transporter A1 (ABCA1) promotes the release of excess cellular cholesterol to apolipoprotein A-I (apoA-I) in the process of forming high density lipoprotein (HDL) particles. To determine whether accumulation of cellular unesterified cholesterol in Niemann-Pick type C (NPC) disease or cholesteryl esters in atherosclerotic lesions is due to impaired ABCA1 actions, the regulation and function of ABCA1 in human *NPC1*$^{-/-}$ fibroblasts and smooth muscle cells (SMC), the predominant cell type in arteries, were studied.

Human *NPC1*$^{-/-}$ fibroblasts showed a markedly decreased ability to release radiolabeled cellular cholesterol and phospholipids to apoA-I compared to *NPC1*$^{+/+}$ fibroblasts, however, apoA-I binding to *NPC1*$^{-/-}$ and *NPC1*$^{+/+}$ fibroblasts was similar. Consistent with impaired lipid efflux, basal and cholesterol-stimulated ABCA1 mRNA and protein levels were markedly decreased in *NPC1*$^{-/-}$ fibroblasts compared to *NPC1*$^{+/+}$ fibroblasts. Also consistent with these results, we observed for the first time decreased plasma HDL-cholesterol levels in 17 of 21 (81%) *NPC1*$^{-/-}$ subjects studied. When ABCA1 expression was increased by an exogenous liver X receptor agonist, TO-901317, *NPC1*$^{-/-}$ fibroblasts showed corrected ABCA1 expression, lipid efflux to apoA-I, and HDL particle formation.

Epithelioid SMC, the predominant SMC subtype in atherosclerotic intima, expressed a trace amount of ABCA1 mRNA and protein at basal level and no significant increase in response to cholesterol loading, which correlated with near absence of apoA-I-mediated lipid efflux. Spindle SMC, the typical SMC subtype

in normal arteries, showed increased ABCA1 expression with cholesterol loading, and active apoA-I-mediated lipid efflux. Although ABCA1 transfection or TO-901317 treatment significantly increased normally phosphorylated ABCA1 in the plasma membrane of epithelioid SMC, apoA-I-mediated lipid efflux was not corrected. Epithelioid SMC showed markedly decreased apoA-I binding compared to spindle SMC. No increase of apoA-I binding was seen following transfection of ABCA1 into epithelioid or spindle SMC.

Correction of ABCA1 activity in *NPC1*$^{-/-}$ fibroblasts provides a potential therapeutic mechanism to decrease lipid accumulation in NPC disease. The lack of correlation between ABCA1 expression and binding or lipid efflux by apoA-I suggests that an additional factor or factors necessary for apoA-I-cell binding are lacking in intimal-phenotype epithelioid SMC.

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I am very thankful to my supervisor, Dr. Gordon Francis. His passion for science has motivated me to do good science, and his insights into science guided me to go to the right direction of research. Also, he has never stopped to encourage me over the past five years. The outstanding experience in his lab will be a big addition for the rest of my life

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LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
ABCA7	ATP-binding cassette transporter A7
ABCG1	ATP-binding cassette transporter G1
ABCG4	ATP-binding cassette transporter G4
ACAT	acyl-CoA:cholesterol acyltransferase
ApoA-I	apolipoprotein A-I
ApoE	apolipoprotein E
ApoB100	apolipoprotein B100
BSA	essentially fatty acid-free bovine serum albumin
bHLH	basic helix-loop-helix
cAMP	cyclic adenosine monophoshate
cDNA	complementary deoxyribonucleic acid
CE	cholesteryl esters
CM	chylomicrons
9-cis RA	9-cis retinoic acid
CoA	coenzyme A
DMEM	Dulbecco's modified Eagle's medium
DMEM/BSA	DMEM containing 1 mg/ml BSA
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FBS	fetal bovine serum
hABCA1 Tg	human ABCA1 expressing transgenic
HASMC	human aortic smooth muscle cells
HITA2	human internal thoracic artery 2
HDL	high density lipoprotein
HDL-CE	HDL-cholesteryl esters
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
Insigs	insulin-induced genes

JAK2	Janus Kinase 2
KO	knockout
LDL	low density lipoprotein
LRP	LDL receptor-related protein
LXR	liver X receptor
mRNA	messenger ribonucleic acid
NPC1	Niemann-Pick type C1
PBS	phosphate-buffered saline
PBS/BSA	PBS containing 1 mg/ml BSA
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PKA	protein kinase A
PKC	protein kinase C
PL	phospholipids
RIPA	radio-immunoprecipitation assay
RCT	reverse cholesterol transport
RXR	retinoid X receptor
SD	Sprague-Dawley
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM	sphingomyelin
SMC	smooth muscle cells
SRE	sterol regulatory element
SREBP	SRE binding protein
SCAP	SREBP cleavage-activating protein
SR-BI	scavenger receptor class B, type I
TBS	Tris-buffered saline
TBS/T	TBS containing 0.1% Tween 20
TG	triglycerides
UC	unesterified cholesterol
VLDL	very low density lipoprotein

WKY	Wistar-Kyoto

CHAPTER 1:

Introduction

Cholesterol is an essential molecule for the normal function of most eukaryotic cells and its cellular distribution, trafficking, and flux are strictly regulated. High density lipoproteins or HDL are the body's main agents for removing excess cellular cholesterol. In this thesis I will present the work I have done studying two different conditions where the formation of HDL and removal of excess cell cholesterol are impaired, Niemann-Pick type C disease and intimal-phenotype smooth muscle cells. The introduction will begin with general information regarding cholesterol. This is followed by an in-depth discussion regarding previous HDL studies that have led to the hypotheses of this thesis.

1.1 Cholesterol

1.1.1 History of cholesterol research

The French chemist Michel Chevreul is credited with the initial discovery of cholesterol in 1815 [1]. He found a substance with fat-like properties in human gallstones and suggested to name it as cholesterine (Greek: *chole*, bile + *stereos*, solid) at a meeting of the French Academy of Sciences [1]. Later it became called cholesterol. As early as 1913, the connection between cholesterol and atherosclerosis was suggested by Anitschkow and his colleagues [2]. Heinrich Wieland was awarded the Nobel Prize in Chemistry 1927 for his studies on the structure of cholesterol and bile acids, and he elucidated the structure of cholesterol in 1932. Cholesterol appeared in medical text books in the 1940s, and the biosynthetic pathway of cholesterol was studied intensively in the 1950s. Most of the crucial steps in the complex pathway were worked out by four biochemists – Konrad Bloch, Feodor Lynen, John Cornforth, and George Popjak – and the major outlines of the pathway were completed by 1960. Konrad Bloch and Feodor Lynen were awarded the Nobel Prize in Physiology or Medicine in 1964 for their discoveries concerning the mechanism and regulation of cholesterol and fatty acid metabolism. In 1974, Michael Brown and Joseph Goldstein found that the level of low density lipoprotein (LDL) cholesterol in the blood is controlled by the activity of cell-surface LDL receptors, and that familial hypercholesterolemia is caused by genetic defects in the LDL receptor, leading to impaired removal of LDL cholesterol from the blood. Brown and Goldstein were awarded the Nobel Prize in Physiology or Medicine 1985 for their discoveries concerning the regulation of cholesterol metabolism. Today, blood cholesterol levels are recognized as among the strongest predictors of the pathogenesis of atherosclerosis, and many cholesterol-lowering drugs are in clinical use or in development for the treatment of atherosclerosis. Recent research has also emphasized the critical role of cholesterol in the development and function of the brain.

1.1.2 Structure of cholesterol

Cholesterol ($C_{27}H_{46}O$) consists of the core steroid rings (A, B, C, and D), a polar hydroxyl head group on C3, and a non-polar hydrocarbon tail at C17 (Figure 1-1A). The side (Figure 1-1B) and top view (Figure 1-1C) show how flat and straight cholesterol appears. The steroid core and the hydrocarbon tail make cholesterol lipid soluble, and possession of the hydroxyl group makes cholesterol a steroid alcohol or "sterol" and minimally soluble in water. Cholesterol is found in both leaflets of the plasma membrane and other membranes, and the polar hydroxyl head group is aligned with the polar head of the phospholipids (PL) (Figure 1-1D).

1.1.3 Biological functions of cholesterol

Cholesterol, present as an unesterified free molecule or esterified to fatty acid in the form of cholesteryl esters (CE), is the most abundant steroid and accounts for ~99% of all sterols in mammals. Cholesterol is crucial for the function of most eukaryotic cells and plays multiple biological roles (Figure 1-2). Cholesterol is a major structural component of cellular membranes, and biophysical studies have demonstrated that it modulates membrane fluidity and ion permeability [3]. Cholesterol, together with sphingolipids (sphingomyelin and glycosphingolipids), constitutes membrane microdomains called lipid rafts in the outer leaflet of the plasma membrane [4]. Lipid rafts incorporate distinct classes

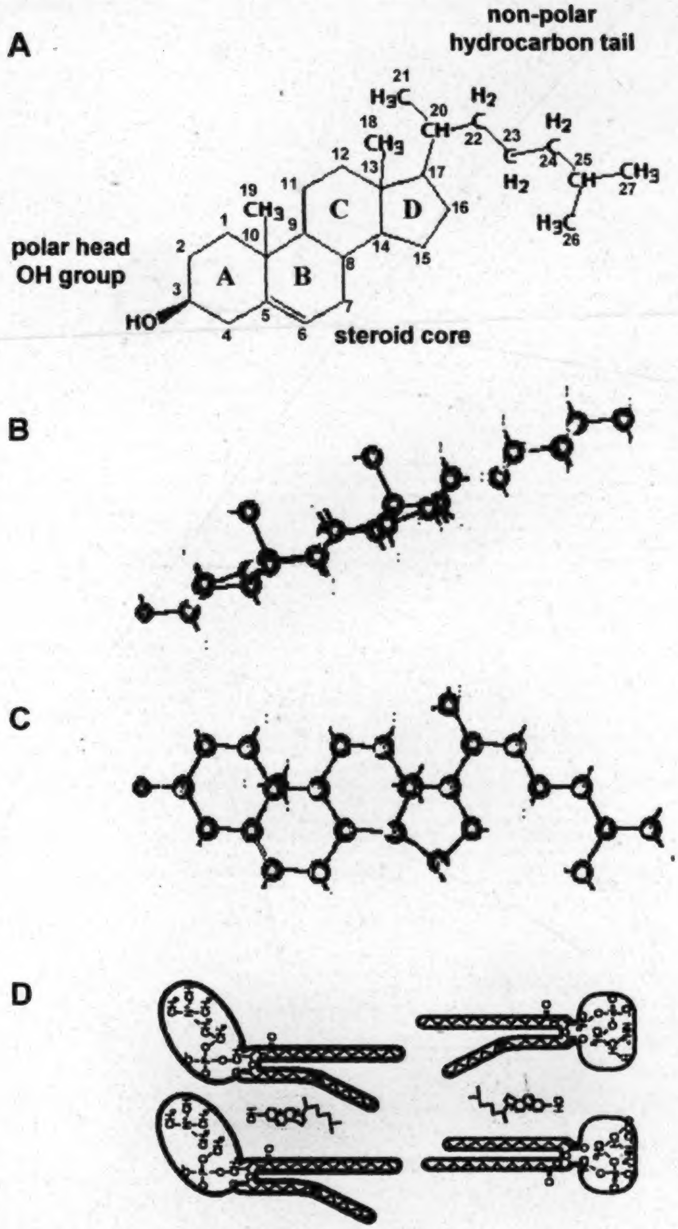

A
non-polar
hydrocarbon tail
21
H_3C
20
H_2
18
CH
22
23
H_2
CH_3
12
13
17
24
25
H_2
CH
CH_3
27
19
11
CH_3
9
C
D
16
1
10
H_3C
26
polar head
OH group
2
14
A
B
8
15
3
7
HO
5
4
6
steroid core
B
C
D

Figure 1-1. Structure of cholesterol. (A) Line-bond structure with numbering of carbon atoms in pink. Ball-and-stick structure from the side **(B)** or top view **(C)** showing the polar head OH group in red. **(D)** Alignment of cholesterol between phospholipid molecules in the plasma membrane. Figure 1-1D was adapted from K. Simons and E. Ikonen [4] with permission.

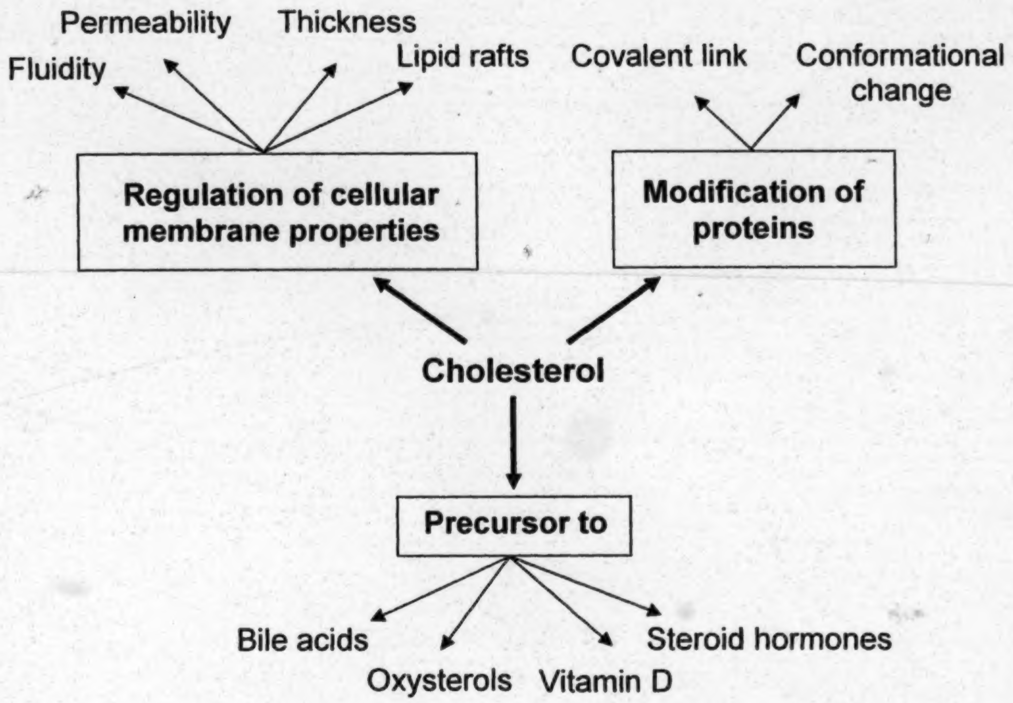

Permeability
Thickness
Fluidity
Lipid rafts
Covalent link
Conformational change
Regulation of cellular membrane properties
Modification of proteins
Cholesterol
Precursor to
Bile acids
Steroid hormones
Oxysterols
Vitamin D

Figure 1-2. Biological functions of cholesterol in eukaryotic cells.

of proteins and are involved in signal transduction [5]. It was also proposed that cholesterol together with sphingolipids facilitates protein sorting in the Golgi apparatus membrane by regulating the thickness of the lipid bilayer [6]. In addition to being the essential component in cellular membranes, cholesterol is the precursor for the synthesis of bile acids in the liver, oxysterols in most tissues, vitamin D in the skin, and steroid hormones in the adrenal glands, ovaries and testes [7]. Cholesterol is also involved in modification of proteins. The covalent link of cholesterol to *Drosophila* Hedgehog protein is crucial for its targeting and function [8, 9]. Direct binding of cholesterol to sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) changes the conformation of SCAP and allows it to bind to insulin-induced genes (Insigs). This leads to retention of the SCAP/SREBP complex in the endoplasmic reticulum (ER), thereby preventing activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) and LDL receptor genes by the cleaved form of SREBP in the nucleus, and slowing cholesterol synthesis and uptake [10, 11].

SCAP is one of seven membrane proteins presently known that have a sterol-sensing domain. All of the seven proteins have some relation to cholesterol metabolism and include the rate-limiting cholesterol biosynthetic enzyme HMG-CoA reductase [12], the cholesterol biosynthetic enzyme 7-dehydrocholesterol reductase [13], the Niemann-Pick type C1 (NPC1) protein that is thought to facilitate the transport of cholesterol and other lipids from late endosomes/lysosomes to destinations throughout the cell [14, 15], the Niemann-Pick type C1-like 1 protein that facilitates the intestinal absorption of dietary cholesterol [16], the developmental protein Patched whose ligand is Hedgehog mentioned above [17], and the plasma membrane protein Dispatched that facilitates the release of cholesterol-modified Hedgehog from Hedgehog-producing cells [17]. Direct binding of a cholesterol analogue to NPC1 has also been reported [18]. It will therefore be intriguing whether the functions of other membrane proteins sharing the sterol-sensing domain are also dependent on direct binding of cholesterol.

1.1.4 Cholesterol homeostasis

Although cholesterol plays an essential role in normal cell structure and function, over-accumulation of cholesterol causes cytotoxicity and many diseases. Because of this, normal cells tightly regulate cholesterol levels to satisfy the balance between demand and supply. Mammalian cells keep cellular cholesterol levels tightly balanced by regulating biosynthesis, influx, and efflux of cholesterol.

1.1.4.1 Biosynthesis of cholesterol

As shown in Figure 1-3, cholesterol in mammals is synthesized from the two-carbon building block, acetyl-CoA. Two molecules of acetyl-CoA are condensed to acetoacetyl-CoA by acetoacetyl-CoA thiolase, and then HMG-CoA synthase condenses acetoacetyl-CoA with another molecule of acetyl-CoA to

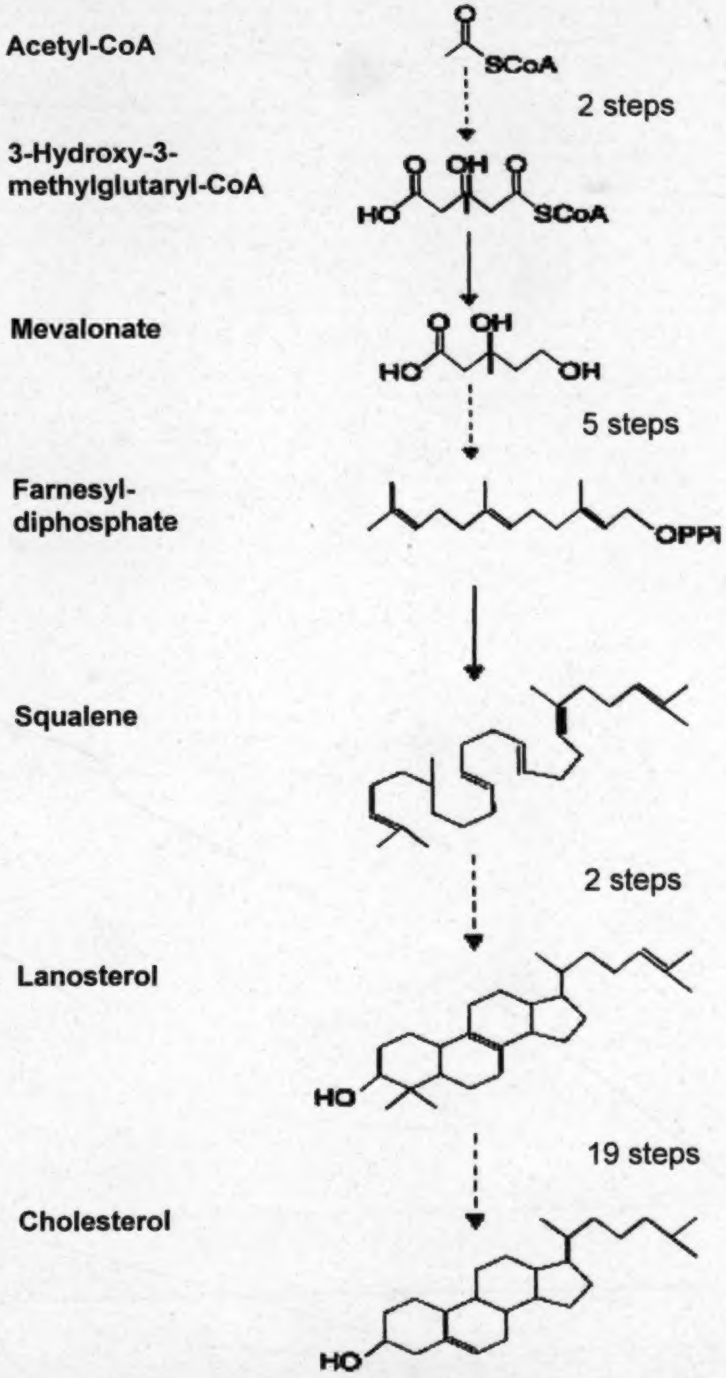

Acetyl-CoA
O
SCoA
2 steps
3-Hydroxy-3-methylglutaryl-CoA
O
OH
O
HO
SCoA
Mevalonate
O
OH
HO
OH
5 steps
Farnesyl-diphosphate
OPPi
Squalene
2 steps
Lanosterol
HO
19 steps
Cholesterol
HO

Figure 1-3. The cholesterol biosynthetic pathway in mammalian cells. The major intermediates and cholesterol are depicted. The figure was adapted from T.V. Kurzchalia and S. Ward [7] with permission.

form HMG-CoA. HMG-CoA reductase catalyzes the reduction of HGM-CoA to mevalonate utilizing two molecules of NADPH. HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthetic pathway and is highly regulated by the cellular cholesterol content (reviewed in [19]). The steady state levels of HMG-CoA reductase can be altered 200-fold by the combined regulation of synthesis and turnover. Mevalonate is metabolized to farnesyldiphosphate by five enzymatic reactions. Two farnesyldiphosphates are condensed head to head by squalene synthase to form squalene, which is the first committed step in cholesterol synthesis. Squalene synthase is highly regulated by cellular cholesterol content. Farnesyl diphosphate can also be converted into non-steroidal isoprenoids such as prenylated proteins, heme A, dolichol, and ubiquinone. Squalene synthase therefore plays an important role in directing the metabolic flow of farnesyl diphosphate into the sterol or non-sterol branches of the pathway. Squalene is converted into the first sterol, lanosterol, by the action of squalene epoxidase and oxidosqualene cyclase. Lanosterol is then converted to cholesterol by a series of oxidation, reduction, and demethylation steps.

The expression levels of HMG-CoA reductase and many other cholesterol-responsive genes are strongly regulated by cellular cholesterol content due to having a similar sequence termed the sterol regulatory element (SRE) within their promoter regions [20-22]. SRE has been identified at least in HMG-CoA synthase, HMG-CoA reductase, farnesyldiphosphate synthase, and squalene synthase genes. The transcriptional factor termed the SRE-binding protein (SREBP) binds the SRE and activates transcription. There are two SREBP genes that produce three isoforms. SREBP-1a and -1c are produced from a single gene on human chromosome 17p11.2 [23]. SREBP-2 is produced from a separate gene on human chromosome 22q13 and is the major transcription factor regulating cholesterol biosynthesis [23]. SREBPs play a fundamental role in both cholesterol homeostasis and fatty acid metabolism by regulating more than 30 genes involved in the synthesis of cholesterol, fatty acids, triglycerides (TG), and PL [23, 24]. The current model of cholesterol-mediated SREBP regulation is illustrated in Figure 1-4. When cellular cholesterol levels are

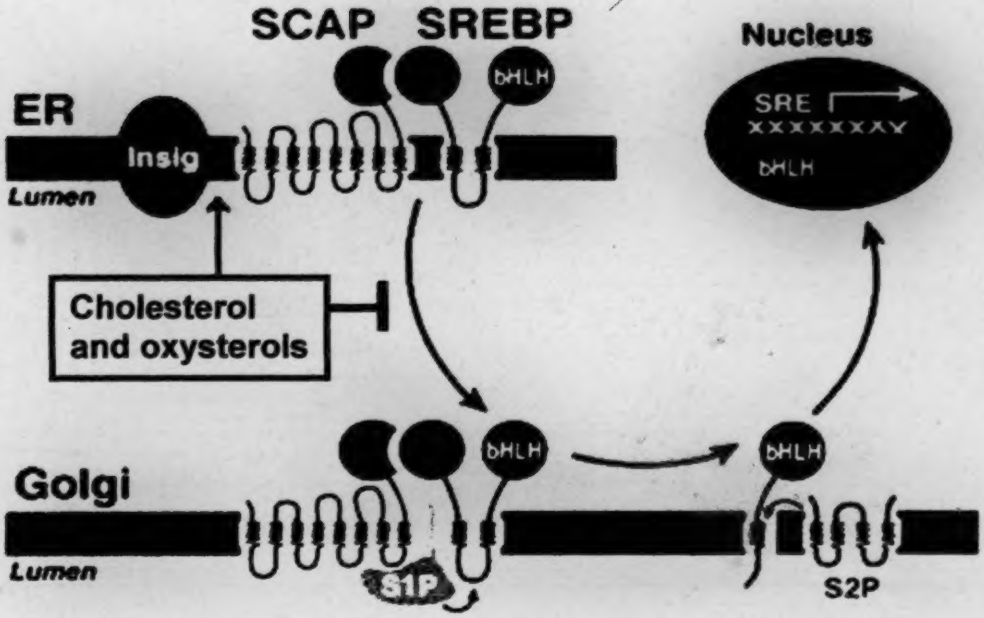

SCAP
SREBP
Nucleus
ER
Insig
bHLH
Lumen
SRE
bHLH
Cholesterol and oxysterols
Golgi
bHLH
bHLH
Lumen
S1P
S2P

Figure 1-4. Current model of sterol-mediated regulation of the SREBP pathway. When cellular cholesterol levels are low, SCAP escorts SREBP from the ER to the Golgi where the N-terminal bHLH-zipper domain of SREBP is released into the cytoplasm by S1P cleavage of a luminal loop followed by S2P cleavage within a transmembrane span. The released SREBP transcription factor migrates to the nucleus and activates transcription of SRE-containing target genes. When cellular cholesterol levels are high, cholesterol and oxysterols elicit the binding of SCAP to Insig, resulting in the retention of the SCAP/SREBP complex in the ER membrane. The figure was adapted from the website of Brown-Goldstein laboratory (http://www8.utsouthwestern.edu/utsw/cda/dept14857/files/114532.html) with permission.

low, the SREBP precursor is synthesized and inserted into the ER membrane and escorted from the ER to the Golgi by a chaperone, SCAP, where the 68-kDa N-terminal basic helix-loop-helix (bHLH) zipper domain of SREBP is proteolyzed by the site-1 protease (S1P) and the site-2 protease (S2P), and then is translocated into the nucleus to activate transcription of SRE-containing target genes. When cellular cholesterol levels rise to a threshold level, cholesterol binds directly to SCAP and induces a conformational change, which allows SCAP to bind to ER retention proteins, Insigs, resulting in a blockage of SCAP/SREBP travel from the ER to the Golgi. As a result, the SREBP precursor is not proteolyzed in the Golgi and the transcription of SREBP-target genes declines to basal levels.

Recently, Adams *et al.* [11] reported that 25-hydroxycholesterol was more potent than cholesterol in eliciting SCAP binding to Insigs, but cross-linking of 25-hydroxycholesterol to SCAP and a conformational change in SCAP were not observed. This study suggests that oxysterols generated in cholesterol-loaded cells regulate SCAP/SREBP pathway in a mechanism different from cholesterol-mediated regulation.

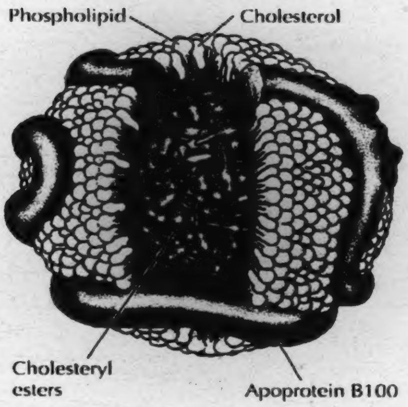
1.1.4.2 Influx of cholesterol

To maintain cholesterol homeostasis, cells also regulate influx of their exogenous source of cholesterol supplied from lipoproteins. Lipoproteins are lipid-protein complexes that transport lipids in the circulation of all vertebrates and even insects. Lipoproteins consist of a core of non-polar lipids including CE and TG, surrounded by a surface monolayer consisting of phospholipids, cholesterol, and proteins termed apolipoproteins. Based on their relative contents of protein and lipid, they are classified generally into chylomicrons (CM), very low density (VLDL), low density (LDL), and high density (HDL) lipoproteins. The liver assembles and secretes TG-rich VLDL particles into the circulation to supply TG and cholesterol to peripheral tissues. Once VLDL particles have lost most of their TG by the action of lipoprotein lipase, they become cholesterol-rich LDL particles. The LDL particles are highest (37-48%) in CE content as % of particle weight, and in humans are the primary carriers of cholesterol for delivery to all non-central nervous system tissues. The simplified structure of LDL is shown in Figure 1-5A.

LDL-cholesterol is taken up by cells via diverse pathways including simple diffusion and receptor-mediated uptake [25, 26]. The best characterized and quantitatively the most important process in eukaryotic cells is receptor-mediated endocytosis of LDL, in which LDL particles are delivered to cells through LDL receptors localized within specialized plasma membrane microdomains, clathrin-coated pits [27]. As depicted in Figure 1-5B, the LDL receptors mediate endocytosis of whole LDL particles, and multiple steps are employed to release LDL-cholesterol from the particles. The exit of LDL-derived cholesterol from endosomes and lysosomes is crucial for subsequent travel to intracellular compartments and involves the NPC1 and NPC2 proteins. Although the exact function of the NPC proteins has not been assigned, mutations in NPC genes lead to the accumulation of cholesterol and other lipids in late endosomes/lysosomes. The NPC proteins are therefore thought to direct the transport of LDL-cholesterol and other lipids out of late endosomes/lysosomes. Details of the NPC proteins will be discussed further in Section 1.3.

LDL cholesterol is not the only exogenous source of cholesterol but is a key factor in the feedback-regulation of cholesterol homeostasis [27]. LDL-derived cholesterol transported to the ER suppresses the cholesterol biosynthetic pathway as shown in Figure 1-4 and activates acyl-CoA:cholesterol acyltransferase (ACAT), which catalyzes the esterification of excess cholesterol for storage in cytoplasmic lipid droplets. This protects cells from the cytotoxicity of excess cholesterol since esterified cholesterol is less cytotoxic than cholesterol. The expression level of the LDL receptor is also suppressed by LDL-cholesterol, since the LDL receptor gene has the SRE sequence within its promoter region and thus is subjected to transcriptional regulation by the SREBP pathway. In this way, the regulation of both cholesterol biosynthesis and the influx of LDL-cholesterol are tightly interconnected and controlled.

A. LDL


Phospholipid
Cholesterol
Cholesteryl esters
Apoprotein B100

B. LDL pathway

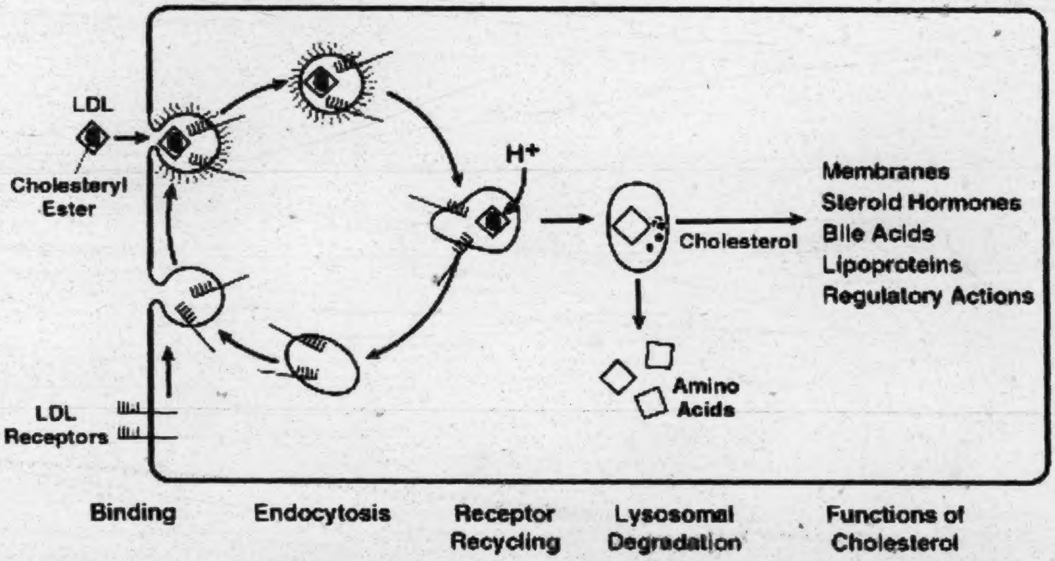

LDL
Cholesteryl Ester
LDL Receptors
H+
Cholesterol
Membranes
Steroid Hormones
Bile Acids
Lipoproteins
Regulatory Actions
Amino Acids
Binding
Endocytosis
Receptor Recycling
Lysosomal Degradation
Functions of Cholesterol

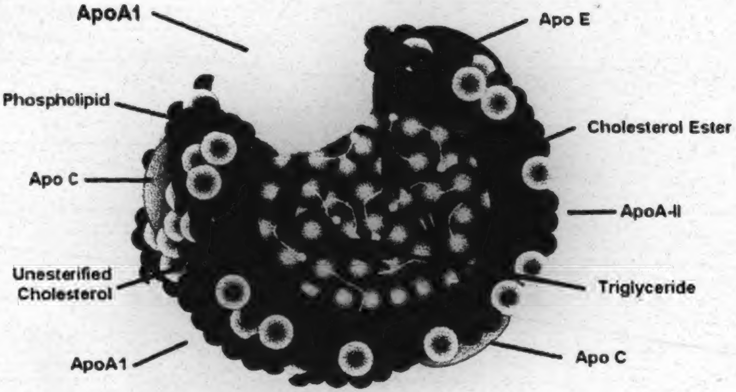
Figure 1-5. The simplified structure of LDL and the receptor-mediated endocytosis of LDL. (A) An LDL particle is 20-25 nm in diameter and apolipoprotein B100 (apoB100) comprises its protein coat. **(B)** LDL particles bind to LDL receptors clustered in clathrin-coated pits. The receptor-LDL complex is internalized in coated vesicles, which become uncoated and acidified by protons (H^+) pumped into their lumen, resulting in endosomes in which the LDL particles dissociate from the receptors due to the low pH. The LDL particles are delivered to lysosomes, but almost all of the receptors recycle to the cell membrane via recycling vesicles. In lysosomes, apoB100 is digested and CE are hydrolyzed, releasing cholesterol for subsequent diverse intracellular actions. Figure 1-5B was obtained from the website of Brown-Goldstein laboratory (http://www8.utsouthwestern.edu/ utsw/cda/dept14857/files/114532.html) and is presented here with permission.

LDL receptor-related protein (LRP), another member of the LDL receptor family, binds and internalizes apolipoprotein E (apoE)-containing lipoproteins such as CM remnants, lipoproteins that shuttle primarily dietary cholesterol from the gut to the liver, and VLDL remnants [28, 29]. LRP is highly expressed not only in the liver, but is also present in virtually all neurons in the brain. The blood-brain barrier prevents lipoprotein transport into the central nervous system [30]. Glial cells in the central nervous system produce apoE-rich lipoproteins that are similar to HDL in size and density [30]. It is therefore thought that LRP may play a major role in mediating the neuronal uptake of glial cell-derived HDL and cholesterol. Recent studies also suggest that LRP has several other functions in brain, including cellular signal transduction, neurotransmission, and permeability control of the blood-brain barrier [31, 32].

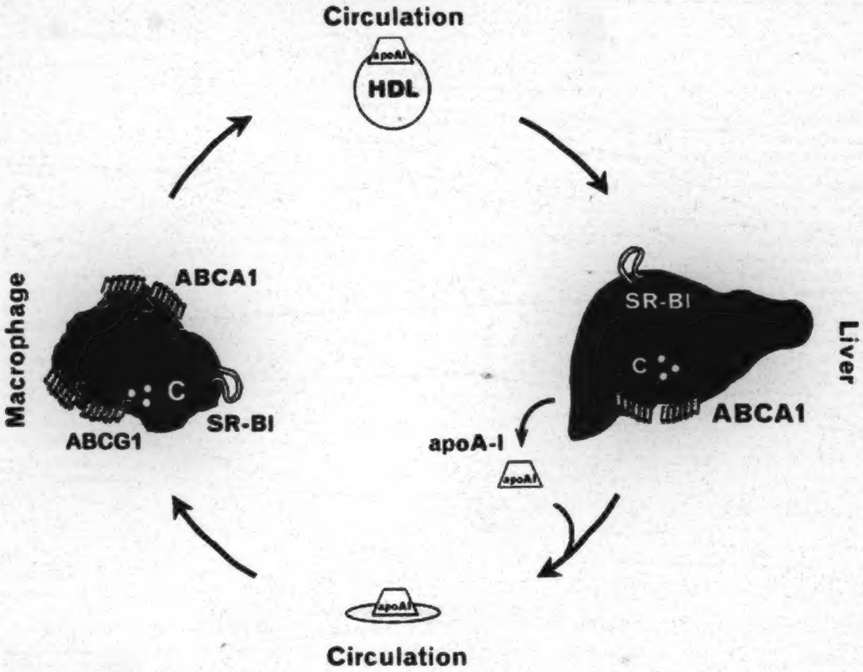
1.1.4.3 Efflux of cholesterol

Another important mechanism to maintain cellular cholesterol homeostasis is the removal of excess cholesterol from cells. As cellular cholesterol levels increase, cells not only suppress new cholesterol synthesis and LDL uptake, they also activate cholesterol efflux pathways by generating oxysterols from excess cholesterol. Oxysterols inhibit the SREBP pathway [11], and also activate the cholesterol efflux pathway by inducing the expression of multiple proteins involved in cholesterol efflux [33]. Cholesterol efflux from peripheral cells and delivery of the cholesterol back to the liver for recycling or excretion into bile are mediated by HDL and its apolipoproteins in concert with several proteins including ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1), and scavenger receptor class B, type I (SR-BI), in a process called reverse cholesterol transport (RCT) [34, 35]. The structure of HDL and a simplified model of RCT are depicted in Figure 1-6. It is believed that a cellular lipid transporter, ABCA1, promotes the RCT pathway by mediating the initial lipidation of lipid-free or lipid-poor apolipoprotein A-I (apoA-I), to generate small HDL that migrate in the pre-β position on agarose gel electrophoresis [36]. The liver secretes lipid-free/poor apoA-I [37, 38] and expresses high levels of ABCA1 [39, 40], suggesting that the liver itself might mediate the initial lipidation of apoA-I. The importance of hepatic ABCA1 in the phospholipidation of newly synthesized apoA-I was reported in a study using primary mouse hepatocytes [41]. Studies using genetically engineered mice have further supported the role of hepatic ABCA1 in HDL formation; a liver-specific deletion of ABCA1 in mice dramatically decreased plasma HDL levels [42], whereas adenoviral over-expression of hepatic ABCA1 increased HDL production [43, 44]. Although it is still controversial how much lipid-free/poor apoA-I exists in human plasma, its presence has been reported [45-47]. It is therefore thought that the liver produces lipid-free/poor apoA-I and partially lipidated apoA-I termed discoidal HDL particles, containing two or three molecules of apoA-I plus PL with or without cholesterol, that migrate in the pre-β position on agarose gel electrophoresis [36]. These cholesterol-poor lipid acceptors circulate to the periphery and acquire cholesterol and PL from peripheral cells such as macrophages in the arterial wall via several mechanisms involving ABCA1, ABCG1, and possibly SR-BI, and thus become cholesterol-rich HDL particles. The particles are then subjected to multiple reactions in the plasma to form spherical HDL particles, containing two or more molecules of apoA-I plus PL, cholesterol, CE and TG, that migrate in the α position on agarose gel electrophoresis [36]. Cholesterol in spherical HDL particles is then proposed to be delivered to the liver via SR-BI, which completes a cycle of the RCT pathway.

ABCA1 interacts preferentially with lipid-free/poor apoA-I to mediate efflux of cellular cholesterol and PL, but it shows very little or no interaction with spherical HDL particles [48]. The critical role of ABCA1 in HDL metabolism has been directly demonstrated by its naturally occurring mutations and animal studies. ABCA1 deficiency in human, mouse, and chicken results in extremely low plasma HDL levels [49-53], whereas transgenic mice overexpressing ABCA1 show raised plasma HDL levels [54, 55]. These facts imply that ABCA1-dependent lipid efflux to lipid-free/poor apoA-I is the first and the most crucial


A. HDL

ApoA1
Apo E
Phospholipid
Cholesterol Ester
Apo C
ApoA-II
Unesterified Cholesterol
Triglyceride
ApoA1
Apo C


B. Reverse cholesterol transport

Circulation
apoAI
HDL
Macrophage
ABCA1
C
ABCG1
SR-BI
SR-BI
C
Liver
ABCA1
apoA-I
apoAI
apoAI
Circulation

Figure 1-6. The structure of HDL and a simplified model of reverse cholesterol transport. (A) An HDL particle is 8-12 nm in diameter and contains about 70% by protein weight of apoA-I, 20% of apoA-II, and 10% of the minor apolipoproteins. **(B)** The liver secretes lipid-free/poor apoA-I and partially-lipidated apoA-I (discoidal HDL) via ABCA1 into the circulation. Peripheral cells such as macrophages in the arterial wall release cholesterol and PL to the cholesterol-poor apoA-I particles, and thus convert them into cholesterol-rich

HDL particles. Several proteins including ABCA1, ABCG1, and SR-BI have been implicated in cholesterol efflux from cells to apoA-I and HDL, with the precise mechanism remaining to be determined. After a series of reactions in the plasma, HDL-cholesterol is delivered back to the liver via SR-BI, leaving lipid-poor apoA-I for further cycling in the reverse cholesterol transport pathway. Figure 1-6B was adapted from M. Van Eck *et al.* [35] with permission.

step in the biogenesis of HDL particles. The detailed role of ABCA1 in HDL metabolism will be discussed in Section 1.2.

ATP-binding cassette transporter A7 (ABCA7), expressed in most tissues tested and sharing 50% sequence identity with ABCA1, has also been reported to mediate efflux of choline-containing PL and possibly a small amount of cholesterol to lipid-free apoA-I but not spherical HDL [56, 57]. Correlated with these findings, Hayashi et al. [58] reported that ABCA1 generates large cholesterol-rich and small cholesterol-poor HDL particles, whereas ABCA7 generates mostly small cholesterol-poor HDL particles. However, knockdown of ABCA7 in mouse peritoneal macrophages using small interfering RNA showed no effect on either PL or cholesterol efflux to lipid-free apoA-I compared with control cells [57]. Consistent with this, bone marrow-derived macrophages isolated from ABCA7-knockout (KO) mice showed normal PL and cholesterol efflux to lipid-free apoA-I compared with wild type control cells [59]. Plasma lipid profiles in male ABCA7-KO mice were similar to those in gender matched littermates, but female ABCA7-KO mice had significantly lower plasma total and HDL cholesterol levels than gender matched littermates even though plasma apoA-I levels were similar to the controls [59]. These findings suggest that ABCA7 may have a gender specific role in lipoprotein metabolism, but does not play an essential role in cholesterol efflux to apoA-I, and does not substitute for the actions of ABCA1.

ABCG1 has been reported to mediate cholesterol efflux to spherical HDL particles but not to lipid-free/poor apoA-I in studies using human embryonic kidney cells overexpressing ABCG1 or peritoneal macrophages isolated from wild type and ABCG1-KO mice [60, 61]. However, both ABCG1-KO and -

transgenic mice had normal plasma lipids and HDL levels even when fed a high-cholesterol diet [61]. These studies suggest that ABCG1 plays some role in mediating cholesterol efflux from macrophages to spherical HDL but is not a major determinant of plasma HDL cholesterol levels.

SR-BI mediates the uptake of spherical HDL-cholesteryl esters (HDL-CE) by cells, through a non-endocytic mechanism in which the cell-surface receptor SR-BI binds spherical HDL particles and mediates the selective transport of spherical HDL-CE to the cell without whole particle uptake [62, 63]. CE-poor HDL particles are then released from the cell surface [62, 63]. Another model of SR-BI action is a retroendocytosis mechanism in which the complex of SR-BI and HDL undergoes endocytosis to release HDL-CE and possibly unesterified cholesterol, followed by re-secretion of cholesterol-poor HDL particles [64, 65]. Although the relative contribution of each mechanism is currently unknown, the important role of SR-BI in the RCT pathway as a cell surface receptor for spherical HDL has been demonstrated by animal studies. Hepatic over-expression of SR-BI in mice decreased plasma HDL levels, and increased both hepatic HDL cholesterol uptake and biliary cholesterol content [66-68], whereas disruption of the SR-BI gene in mice increased plasma cholesterol levels due to the accumulation of abnormally large HDL particles [69, 70].

In addition to its role in the uni-directional uptake of HDL-CE, SR-BI has also been shown to stimulate the bi-directional flux of cholesterol between cells and HDL particles with the direction of flux being determined by the cholesterol concentration gradient [71-73]. Cell culture studies *in vitro* have shown that SR-BI transfected Chinese hamster ovary (CHO) cells have significantly enhanced cholesterol efflux ability to HDL, and that the rate of cholesterol efflux to HDL is positively correlated with SR-BI expression levels in several different cell types including macrophages [71]. The physiological role of macrophage SR-BI in cholesterol efflux, however, is not clear yet. Bone marrow transplantation studies from three different laboratories have shown that selective disruption of SR-BI in bone marrow-derived cells including macrophages did not alter plasma lipoprotein profiles [74-76]. Van Eck *et al.* [75] showed 20% reduction of cholesterol efflux from SR-BI-deficient macrophages to HDL for 24 h compared with control macrophages, whereas Zhang *et al.* [76] did not find any significant effect of macrophage SR-BI expression level on cholesterol efflux to HDL. The net bi-directional flux of cholesterol between cells and HDL might be strongly influenced by cellular cholesterol content. Therefore, SR-BI-mediated cholesterol efflux from macrophages to HDL might be dependent on *in vitro* culture conditions and, *in vivo*, on the stage of atherosclerotic lesion development. It could be currently concluded that hepatic SR-BI-mediated uptake of HDL-CE is essential for the final step of RCT and the recycling of apoA-I/small HDL in the RCT pathway, and that SR-BI-mediated cholesterol efflux from peripheral cells to HDL is dependent on the local environment.

Cellular cholesterol can also pass between the cell membrane and HDL in a mechanism called passive diffusion, in which cholesterol flux is driven by the cholesterol concentration gradient [77, 78] just like SR-BI-mediated bi-directional cholesterol flux. At this time, it is not clear whether the passive diffusion mechanism is distinct from SR-BI actions or is facilitated by other membrane proteins, or does not require membrane proteins.

Although the four proteins ABCA1, ABCA7, ABCG1, and SR-BI might interact at several steps of lipid metabolism, each protein also has a distinct role in HDL metabolism such as specificity to lipid acceptors and direction of cholesterol transport mentioned above. At the current time, a decisive role of ABCA1 in HDL formation is evident based on genetic studies, although the mechanism of ABCA1 action is not fully understood.

1.2 ABCA1-dependent lipid efflux and HDL metabolism

1.2.1 Discovery and structure of ABCA1

ABCA1 was discovered from the study of a rare HDL deficiency syndrome found originally in an isolated community on Tangier Island in Chesapeake Bay, MD, USA. Tangier patients, characterized by extremely low plasma HDL and apoA-I levels and accumulation of cellular CE, have large orange tonsils, peripheral neuropathies, hepatosplenomegaly, and coronary heart disease [79].

In 1999, four different groups identified ABCA1 as the defective gene in Tangier disease patients, and proposed the critical role of ABCA1 in cholesterol efflux to generate HDL particles [80-83].

ABCA1 is a member of a large family of ATP-binding cassette transporters that utilize ATP as an energy source to transport a variety of substrates including ions, lipids, and cytotoxins across membranes [84]. ABCA1 is a 2261-amino acid integral membrane protein arranged in two similar halves. Each half has a six transmembrane-spanning domain followed by a nucleotide-binding domain containing two conserved peptide motifs termed Walker A and Walker B. It has been predicted that each half has a large and highly glycosylated extracellular loop, and that these two loops are linked by 1 or more disulfide bonds [85-87] (Figure 1-7).

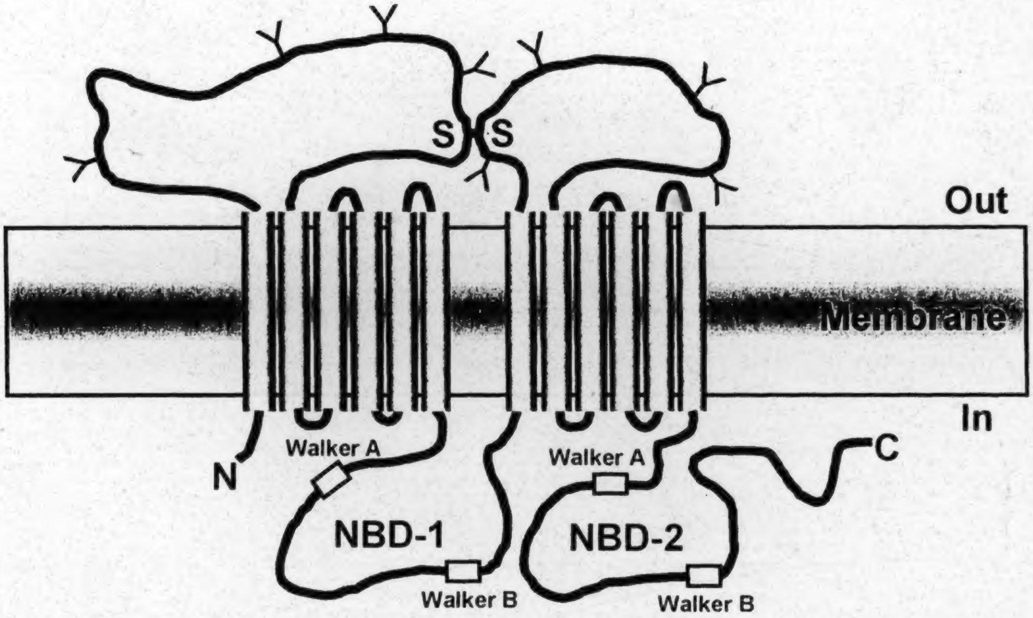


Figure 1-7. Current topological model of ABCA1. "Y" in the extracellular loops indicates approximate glycosylation sites, and S-S indicates 1 predicted disulfide bond. Nucleotide-binding domain 1 (NBD-1) and NBD-2 contain the highly conserved Walker A and Walker B sequences. The figure was obtained from J.F. Oram [87] and is presented here with permission.

1.2.2 Role of ABCA1 in HDL formation

Tangier disease fibroblasts showed little or no ability to release cholesterol and PL to lipid-free apoA-I, however, they retained ability to efflux cholesterol to spherical HDL particles [88]. As mentioned in Section 1.1.4.3, several proteins including ABCG1 and SR-BI are proposed to be involved in HDL-mediated cholesterol efflux, however cholesterol efflux to lipid-free apoA-I is exclusively mediated by ABCA1. When ABCA1-dependent initial lipidation of apoA-I is defective, lipid-free/poor apoA-I will be rapidly removed by the kidney [89-92]. It is therefore believed that the near-absence of plasma HDL in ABCA1 mutation homozygotes (Tangier disease), despite having a normal apoA-I gene and protein, results from the absence of functional ABCA1. An approximately half-normal level of plasma HDL in human ABCA1 mutation heterozygotes has further emphasized the decisive role of ABCA1 in the formation of HDL particles and in determining plasma HDL cholesterol levels [93].

ABCA1-KO mice have been created in three different laboratories to investigate the role of ABCA1 in animal models. McNeish *et al.* [50] and Orso *et al.* [52] used the DBA/1J strain, whereas Christiansen-Weber et al. [51] used the C57BL/6 strain to establish ABCA1-KO mouse lines. Although there were differences between the KO mouse lines in several aspects, including the extent and severity of lipid accumulation in various tissues, survival rate, and other pathological findings, a near-absence of plasma HDL was present in all KO-mouse lines. The differences between the ABCA1-KO mouse lines might be due to different genetic backgrounds and experimental conditions. The common HDL deficiency shown in all the ABCA1-KO mice regardless of these differences demonstrates an intrinsic role of ABCA1 in HDL formation in mice as well as in humans.

Human ABCA1-expressing transgenic mice (hABCA1 Tg-mice) have also been created in three different laboratories. Each hABCA1 Tg-mouse model has unique features including the exon/intron structure of human ABCA1 transgene, the promoter region controlling ABCA1 expression (and thus the sites and levels of ABCA1 expression), and the genetic background of the mice. Vaisman et al.

[54] generated hABCA1 Tg-mice in a pure C57B1/6 background expressing a significant amount of hABCA1 in the liver and macrophages but not in many other tissues that endogenous mouse ABCA1 is expressed. Singaraja et al. [55] generated hABCA1 Tg-mice in a mixed (C57B1/6 × CBAJ) genetic background overexpressing ABCA1 in the liver, macrophages, lungs, small intestine, stomach, testis, and brain. These two hABCA1 Tg-mouse lines showed increased plasma HDL and apoA-I levels when fed both chow and Western-type diets compared with wild type mice. Cavelier *et al.* [94] generated hABCA1 Tg-mice in a pure FVB background. Although these hABCA1 Tg-FVB mice expressed hABCA1 in the liver, macrophages, spleen, lungs, and heart, brain, intestine, kidney, and adrenals [94, 95], the expression level of hABCA1 was low compared with the other two hABCA1 Tg-mouse lines and the mice showed no significant difference in plasma HDL levels compared with-wild type mice fed either chow or Western-type diets. One of the several possible explanations for the insignificant changes in the plasma HDL levels of the hABCA1 Tg-FVB mice could be the lack of sufficient expression of hABCA1. Other possibilities include differences between exogenous hABCA1 and endogenous mouse ABCA1 in actions, effect of other lipoproteins on HDL metabolism, and FVB strain-specific genetic background.

1.2.3 ABCA1-mediated formation of discoidal HDL

The primary function of ABCA1 is believed to be to mediate the delivery of cellular cholesterol and PL to lipid-free/poor apoA-I and thus to form discoidal HDL. The formation of discoidal HDL is therefore thought to be the critical determinant of plasma HDL levels. There are four potential pathways for the formation of discoidal HDL: (1) intracellular assembly in the liver [38, 96]; (2) extracellular formation via ABCA1-dependent lipidation of lipid-free/poor apoA-I; (3) remodeling of HDL in the plasma [97]; and (4) release during lipolysis of CM [98, 99]. Kiss *et al.* [41] suggested a potential involvement of ABCA1 in the 1st pathway by showing that primary hepatocytes from ABCA1-KO mice secrete apoA-I associated with markedly reduced PL content compared with wild-type hepatocytes. The requirement of ABCA1 in the 2nd pathway is obvious, and the

3rd pathway seems to be independent of ABCA1 functions. It is not clear how apoA-I is incorporated onto CM in the intestine, but ABCA1-mediated lipidation of apoA-I might facilitate this step.

The liver is the major organ to synthesize apoA-I [100, 101]. Chisholm *et al.* [38] reported that a mammalian hepatoma cell line, HepG2, secreted ~20% of newly synthesized apoA-I as an intracellularly lipidated form, ~30% in lipid-free/poor form lipidated immediately following secretion, and that the lipidated apoA-I was further lipidated with longer incubation. The authors reported that the lipidated apoA-I particles had a size range of 7.5-8 nm and likely contained two apoA-I molecules, which suggests that HepG2 cells may secrete ~50% of apoA-I in discoidal or spherical HDL form and ~50% in lipid-free/poor form. Considering ABCA1 as a key player in the formation of discoidal HDL, and the liver as the major apoA-I producing organ, hepatic ABCA1-dependent lipidation of the ~50% newly synthesized apoA-I would significantly contribute to plasma HDL cholesterol levels.

The importance of hepatic ABCA1 in determining plasma HDL cholesterol levels has been demonstrated by studies using liver-specific ABCA1-KO or -overexpressing mice. Timminis *et al.* [42] created liver-specific ABCA1-KO mice using the *Cre-loxP* recombination system and observed a marked (~80%) decrease in the plasma HDL cholesterol and apoA-I levels in the KO mice compared with wild type. Basso *et al.* [43] and Wellington *et al.* [44] generated liver-specific ABCA1-overexpressing mice using adenoviral delivery system in the C57BL/6 background. Both studies showed that adenovirus-ABCA1 infected mice have significantly higher plasma HDL cholesterol levels compared with adenoviral-mock infected control mice. Basso *et al.* [43] also showed that lipid-free apoA-I-mediated cholesterol efflux was 2.6-fold higher in primary hepatocytes isolated from adenovirus-ABCA1 infected mice than in control hepatocytes, but no difference in spherical HDL-mediated cholesterol efflux, reemphasizing the role of ABCA1 in the formation of discoidal HDL. The role of hepatic ABCA1 in lipid efflux has been further supported by its localization to the basolateral surface of polarized liver cells [102, 103]. If ABCA1 localized to the apical surface, it would mediate cholesterol efflux to the bile rather than to the plasma. The accumulation of cholesterol in the liver of Tangier patients and in Wisconsin hypoalpha mutant chickens, which have a natural ABCA1 mutation, is consistent with the role of hepatic ABCA1 in promoting cholesterol efflux from the liver [103, 104]. These findings demonstrate that hepatic ABCA1-mediated formation of discoidal HDL plays an important role in hepatic cholesterol homeostasis and in determining plasma HDL cholesterol levels.

The importance of extrahepatic formation of discoidal HDL should not be ignored. Most tissues and cells express ABCA1, and Chisholm *et al.* [38] suggested that the liver may secrete ~50% of apoA-I in lipid-free/poor form. Bone marrow transplantation studies demonstrated that the contribution of macrophage ABCA1-mediated cholesterol efflux to the overall plasma HDL levels is minimal. However, macrophage-specific ABCA1 deficiency results in cholesterol-overloaded macrophage foam cells and the development of atherosclerosis [105-107]. Although the relative contribution of peripheral cells to ABCA1-dependent formation of discoidal HDL has not been determined, the contribution of macrophages must be minimal because they comprise only a very small portion of total peripheral cells in the body, estimated to be approximately 10^8 cells in an adult mouse [108]. Therefore, studies on macrophage ABCA1 strengthen the importance of peripheral ABCA1 by demonstrating the pivotal role of peripheral ABCA1-mediated formation of discoidal HDL in peripheral cholesterol homeostasis. It is also believed that total peripheral cell ABCA1 may significantly contribute to plasma HDL levels.

1.2.4 Mechanism of ABCA1-dependent lipid efflux

While it is generally accepted that ABCA1 promotes the efflux of cellular cholesterol and phospholipids to lipid-free/poor apoA-I, the detailed mechanism of ABCA1 action remains controversial.

1.2.4.1 Plasma membrane versus intracellular lipidation of apoA-I by ABCA1

ABCA1 is primarily localized to the plasma membrane, but it has also been found in the Golgi complex and endocytic vesicles including early endosomes, late endosomes, and lysosomes [52, 102, 109]. Primary localization to the plasma membrane supports the concept that ABCA1 functions mainly at the cell surface to lipidate apoA-I. The presence of ABCA1 in endocytic vesicles might represent ABCA1 pools under processes of degradation. However, several lines of investigation have proposed a retroendocytic mechanism involving endocytosis of an ABCA1/apoA-I complex, lipidation of apoA-I to form HDL, and resecretion of the HDL. This mechanism has been supported by a significant reduction of apoA-I-mediated cholesterol efflux from the murine macrophage RAW264 cell line in the presence of inhibitors of receptor-mediated endocytosis [110]. Smith *et al.* [111] further showed that 8Br-cAMP-induced ABCA1 in RAW264.7 cells markedly increased the uptake of fluorescently labeled apoA-I into intracellular vesicles and increased cholesterol efflux at 37 °C. However, when apoA-I was added to cells at 21 °C, a temperature at which receptor-mediated endocytosis is reduced, there was no increase in the internalization of apoA-I despite an increase in cell surface binding and no significant increase in cholesterol efflux compared with 8Br-cAMP-nontreated control. Neufeld *et al.* [109, 112] suggested that internalized ABCA1/apoA-I-containing endosomes fuse with late endosomes/lysosomes and ABCA1 mobilizes late endosomal/lysosomal lipid pools to apoA-I-associated lipid pools, and lipidated apoA-I in the lumen of the vesicles travels back to the cell surface to be released as HDL particles. Their studies using time-lapse video fluorescence microscopy showed ABCA1-green fluorescent protein (GFP) fusion protein traveled between the cell surface and late endosomal/lysosomal compartments, and that treatment with brefeldin A or monensin, intracellular vesicular traffic blocking agents, trapped the ABCA1-GFP protein in late endosome/lysosome compartments in HeLa cells, resulting in markedly reduced apoA-I-mediated cholesterol efflux [109]. Also, they showed that late endosome/lysosome vesicles in Tangier disease fibroblasts were defective in trafficking and accumulated lipids (cholesterol and sphingomyelin) and protein (NPC1), and that adenovirus-mediated ABCA1-GFP expression corrected the late endosome/lysosome trafficking defects and restored apoA-I-mediated cholesterol efflux [112]. Recently, Chen *et al.* [113] suggested that ABCA1 promotes lipid efflux to apoA-I in late endosomes/lysosomes as well as at the cell surface. They showed that a cytoplasmic PEST sequence-deleted ABCA1, a mutant defective in internalization into late endosomes/lysosomes, has higher ability to remove plasma membrane cholesterol pools to apoA-I but has lower ability to remove late endosomal/lysosomal cholesterol pools compared with wild-type ABCA1 in HEK293 cells.

1.2.4.2 Models of apoA-I-ABCA1 interaction

There are presently three models to explain the interaction between ABCA1 and apoA-I to mediate lipid efflux (Figure 1-8). The first model has proposed that a direct association between ABCA1 and apoA-I is required for the lipidation of apoA-I (Figure 1-8A). This model has been supported by functional cross-linking studies. Four natural mutations in the extracellular loops of ABCA1 abolished its cross-linking and cholesterol efflux ability to apoA-I even though these mutants were localized to the plasma membrane [114]. In addition, cross-linking studies by Chroni *et al.* [115] indicated that apoA-I and ABCA1 are within a distance of 3 Å of each other. These studies showed that the cross-linking ability of various apoA-I mutants to ABCA1 is positively correlated with cholesterol efflux. These findings suggest that ABCA1 and apoA-I interact in very close proximity or are directly associated to lipidate apoA-I. A specific mutation (W590S) in the first extracellular loop of ABCA1 had a greater efficiency in cross-linking to apoA-I than wild-type, but the apoA-I was released in a lipid-free form with similar dissociation kinetics to the dissociation of lipidated apoA-I from wild-type ABCA1 [114, 116]. The formation of a non-productive complex of ABCA1 and apoA-I suggests that improper association of the two proteins fails to induce subsequent cellular events such as conformational changes of ABCA1 and/or intracellular signaling events required for the transport of lipids to apoA-I.

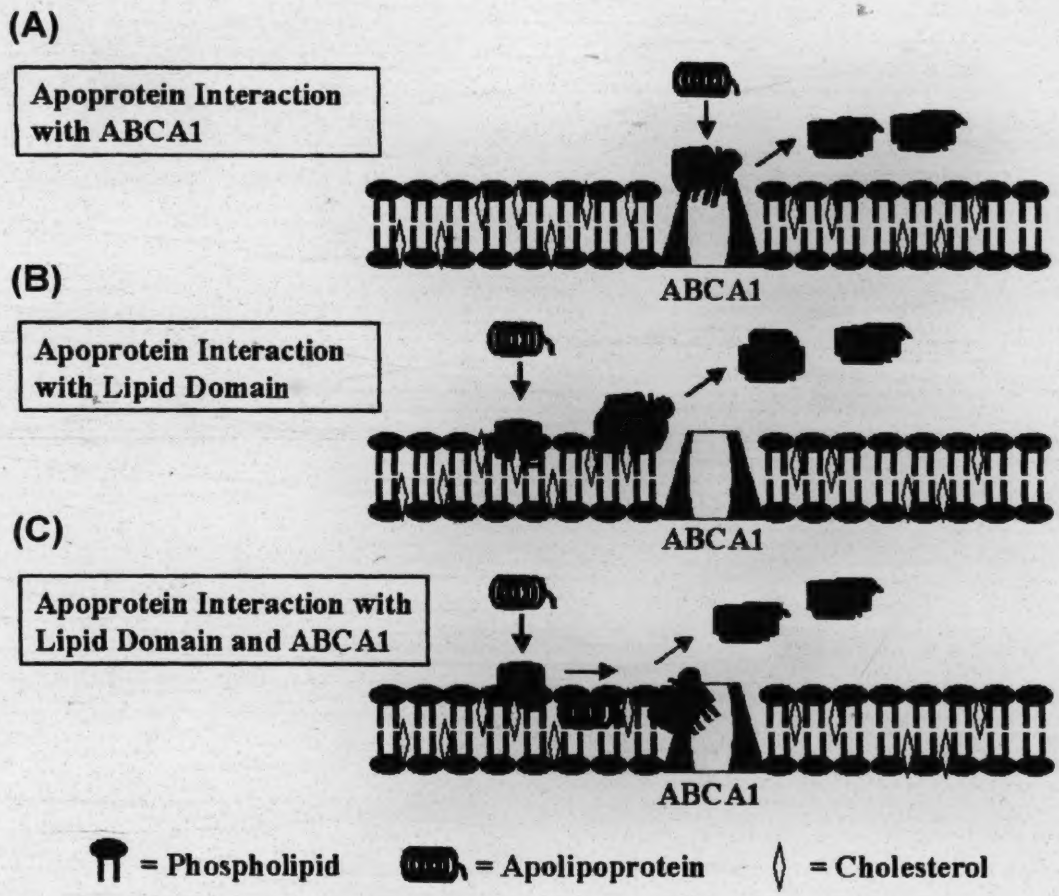

(A)
Apoprotein Interaction with ABCA1
ABCA1
(B)
Apoprotein Interaction with Lipid Domain
ABCA1
(C)
Apoprotein Interaction with Lipid Domain and ABCA1
ABCA1
= Phospholipid
= Apolipoprotein
= Cholesterol

Figure 1-8. Proposed models for ABCA1-mediated lipid efflux to apoA-I. (A) ApoA-I binds directly to ABCA1 and acquires both cholesterol and phospholipids to form HDL. **(B)** ApoA-I associates with a specialized membrane domain formed by the action of ABCA1 and extracts membrane lipids. **(C)** Helix 10 of apoA-I tethers apoA-I to a specialized membrane domain formed by ABCA1. ApoA-I diffuses laterally along the membrane bilayer and complexes with ABCA1 to accept lipids. The figure was obtained from P.G. Yancey *et al.* [78] and is presented here with permission.

ABCA1-dependent lipid efflux is not specific to apoA-I, but can also occur with other apolipoproteins containing amphipathic helices, such as apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE [117, 118] and synthetic amphipathic helical peptides [119, 120] that mimic apoA-I. The lack of an obvious common binding sequence among these amphipathic molecules has led to the second model, in which apoA-I interacts with a specialized membrane lipid domain formed by ABCA1 rather than directly with ABCA1 (Figure 1-8B). In support of this model, Chambenoit *et al.* [121] reported that ABCA1 levels at the surface of RAW264.7 cells are positively correlated with cell surface binding of apoA-I, but postulated that apoA-I associates with specially arranged membrane lipids rather than with a protein. This proposal was based on their observations that the mobility of membrane-associated apoA-I was not consistent with the mobility of ABCA1 on the cell surface and that apoA-I binding at the cell surface was positively correlated with the exposure of phosphatidylserine to the outer leaflet of the plasma membrane. Rigot *et al.* [122] reported that the ABCA1 mutant W590S has an enhanced binding capacity to apoA-I compared with wild-type, but fails to drive both flipping of phosphatidylserine at the plasma membrane and release of PL to apoA-I, suggesting that ABCA1-mediated rearrangement of membrane lipids may be independent of the binding of apoA-I. Consistent with this idea, Vaughan *et al.* [123] reported that overexpression of ABCA1 in baby hamster kidney cells in the absence of apolipoproteins redistributes cellular cholesterol to cell surface domains, where it becomes accessible for removal by apoA-I. Furthermore, Drobnik *et al.* [124] showed that in human skin fibroblasts, apoA-I mediates lipid efflux from distinct membrane microdomains enriched in cholesterol and choline-containing PL where ABCA1 is not found. These findings indicate that functional ABCA1 generates special membrane domains enriched in some PL and cholesterol in the outer leaflet of the plasma membrane, which allows apoA-I to associate with the domains and extract lipids to form HDL.

A third model requiring the interaction of apoA-I with both lipid domains and ABCA1 has been proposed by Panagotopulos *et al.* [125] (Figure 1-8C).

They suggested that the 10th helix of apoA-I, the strongest lipid binding helix in apoA-I [126], tethers apoA-I to lipid domains formed by ABCA1. This suggestion was based on their observations that lipid binding abilities of various helix 10 mutants of apoA-I are positively correlated with their abilities in ABCA1-dependent lipid efflux. In addition, they suggested that the tethered apoA-I may diffuse laterally along the membrane to bind ABCA1 and induce ABCA1-mediated lipidation of apoA-I rather than directly extracting lipids from the binding domains. This suggestion was based on their observations that two helix replacement mutants (H4@H1 and H10@H7; helix 1 and 7 were replaced with the sequence for helix 4 and 10, respectively) exhibited ABCA1-dependent cholesterol efflux capacities similar to wild-type in spite of markedly reduced lipid binding affinity. This third model absolutely requires functional helix 10 for the initial binding of apoA-I to lipid domains. However, Chroni *et al.* [127] showed that the central helices (3-7) of apoA-I are sufficient to promote ABCA1-dependent lipid efflux, despite finding that a helices 3-7 mutant has an impaired ability to stimulate ABCA1-independent lipid efflux pathway *in vitro*. Their *in vivo* studies utilizing adenovirus-mediated gene transfer in apoA-I-KO mice showed that mice infected with wild-type apoA-I formed spherical HDL, whereas mice infected with the helix 3-7 mutant formed discoidal HDL [127]. These data suggest that the strongest (helix 10) and the second strongest (helix 1) lipid binding helices in apoA-I [126] may function after discoidal HDL formation by tethering discoidal HDL to lipid domains for further maturation into spherical HDL. At present, none of the three models is conclusively accepted, and further studies are required to elucidate the interaction mechanism between apoA-I and ABCA1 to generate HDL particles.

1.2.4.3 Lipid mobilization by ABCA1

Although ABCA1 is thought to promote the transport of both cholesterol and PL to apoA-I, its primary substrate and the process of lipid transport are not yet known. Two models have been proposed: a one-step and a two-step process. According to the two-step model, ABCA1 first transports PL to apoA-I to form apoA-I-PL complexes, which then acquire cholesterol in a second ABCA1-independent step. This model seems to be plausible when we consider that the formation of discoidal HDL, which is believed to be the major role of ABCA1, absolutely requires the loading of PL to make a lipid bilayer capable of absorbing/solubilizing cholesterol. The presence of two discrete steps has been supported by several different studies. Fielding *et al.* [128] showed that apoA-I-mediated cholesterol efflux was dramatically reduced by vanadate, while PL efflux was minimally reduced. Moreover, they showed that apoA-I-containing medium preincubated with ABCA1-expressing smooth muscle cells promoted cholesterol efflux but not PL efflux from human aortic endothelial cells that do not express ABCA1. Wang *et al.* [48] reported that depletion of cellular cholesterol by treatment with cyclodextrin abolished ABCA1-dependent cholesterol efflux but PL efflux was not affected. They also found that apoA-I-containing conditioned medium from cyclodextrin pre-treated (to deplete plasma membrane cholesterol)-ABCA1-transfected HEK293 cells was much more efficient in promoting cholesterol efflux from ABCA1-deficient HEK 293 cells, when compared to apoA-I-containing conditioned medium from cyclodextrin pre-treated-mock-transfected HEK293 cells [48]. Arakawa *et al.* [129] showed that undifferentiated THP-1 monocytes formed HDL particles containing PL with almost no cholesterol, whereas differentiated THP-1 macrophages formed HDL enriched in both PL and cholesterol. However, there are no convincing kinetic data yet that support the sequential transport of PL and then cholesterol.

The one-step model proposes that ABCA1 transports both PL and cholesterol to apoA-I simultaneously, and has been supported by kinetic studies. Gillotte *et al.* [130] showed that the efflux rate of PL during the initial 10 min of apoA-I incubation with human skin fibroblasts was similar to that of cholesterol, and that the *K*m value of apoA-I for PL efflux was not significantly different from that for cholesterol efflux. In contrast to the findings of Wang *et al.* [48], Vaughan *et al.* [123] observed that apoA-I-containing conditioned medium from ABCA1-transfected baby hamster kidney (BHK) cells was no more effective than conditioned medium from mock-transfected BHK cells in promoting cholesterol efflux from mock-transfected BHK cells that are deficient in ABCA1 expression, regardless of whether cells were pretreated with cyclodextrin. These observations indicate the expression of ABCA1 is required to promote cholesterol efflux even after formation of apoA-I-PL complexes. In addition, Smith *et al.* [111] showed that apoA-I-containing conditioned medium from ABCA1-expressing RAW264.7 or ABCA1-transfected HEK293 cells did not promote cholesterol efflux when the medium was incubated with RAW264.7 or HEK293 cells that lacked ABCA1 expression, regardless of whether cells were pre-treated with cyclodextrin. Also, the authors reported that the high concentration of vanadate (1 mM) and cyclodextrin (20 mM) used by Fielding et al. [128] and Wang et al. [48], respectively, increases PL efflux from ABCA1-induced RAW264.7 cells even in the absence of apoA-I, suggesting that PL efflux measured by Fielding et al. [128] and Wang et al. [48] might be not derived from an apoA-I-dependent mechanism.

The process of lipid transport will also be dependent upon how ABCA1 interacts with apoA-I, and where ABCA1 functions, as mentioned above. At present, there is no dominant model that can reconcile all the different findings. Moreover, there might be tissue and cell specific mechanisms, for instance, apoA-I synthesized by the liver might be lipidated before secretion. It is therefore conceivable that more than one mechanism of ABCA1-dependent lipid efflux exists to meet diverse functional demands.

1.2.5 Expression and regulation of ABCA1

ABCA1 is expressed in most of tissues and cells, with high levels in macrophages, liver cells, intestinal cells, adrenal gland, and placental trophoblasts [39]. ABCA1 protein turns over rapidly with a half-life of 1-2 h [131, 132], which suggests that ABCA1 level is strictly controlled and is metabolically unstable like cell-cycle regulators [133], and that transcriptional regulation plays a key role in maintaining ABCA1 levels.

The transcription of ABCA1 is regulated by various molecules including cholesterol, nutritional lipids, hormones, and cytokines, as reviewed by Schmitz and Langmann [134]. Considering the critical role of ABCA1 in cholesterol efflux, regulation in response to cellular cholesterol levels seems to be most important. Cells overloaded with cholesterol stimulate the transcription of ABCA1 [135]. This inductive effect of cholesterol is mediated by activation of the nuclear receptors liver X receptor α and β (LXRα and LXRβ) [135, 136]. LXRα is highly expressed in macrophages and liver with lower, but significant, levels in kidney, spleen, intestine and adipose tissue, whereas LXRβ is expressed ubiquitously [137, 138]. LXR binds as a heterodimer with the retinoid X receptor (RXR) to specific response elements called LXR response elements of target genes, which are comprised of two direct repeats of a hexanucleotide motif (AGGTCA) separated by four nucleotides [139, 140]. Ligands for either LXR or RXR activate a target gene, but when ligands for both receptors are present, there is additive or synergistic activation [135, 136]. Although cholesterol loading of cells induces LXR target genes, LXR ligands are a specific group of oxysterols. Therefore the conversion of cholesterol to oxysterols is required for transcriptional activity. Naturally occurring oxysterols 20(S)-, 22(R)-, 24(S)-, 27-hydroxycholesterol, and 24(S),25-epoxycholestrol have been demonstrated to activate LXR [135, 136, 141-145].

Steroidogenic cells produce 20(S)- and 22(R)-hydroxycholesterol as intermediates in steroid hormone biosynthesis. [142]. 24(S)-hydroxycholesterol is formed in the brain and is also found in the liver [146-148]. The most abundant hydroxycholesterol in human circulation is 27-hydroxycholesterol, and the rate-limiting enzyme in its synthetic pathway, sterol 27-hydroxylase encoded by *CYP27A1* gene, is ubiquitously expressed with the highest level in the liver [145, 149, 150]. One of the most potent oxysterols in activating LXR is 24(S),25-epoxycholestrol [151], formed by a shunt pathway of cholesterol biosynthesis [152, 153]. Thus, this oxysterol might not be physiologically relevant since cholesterol-loaded cells would block the cholesterol synthetic pathway and therefore the generation of 24(S),25-epoxycholestrol. Consistent with this concept, Fu *et al.* [145] observed that cholesterol-loaded macrophages produce 27-hydroxycholesterol but not 24(S),25-epoxycholestrol.

Vitamin A (retinol), derived from carotinoids in plants and retinyl esters in animal fat, is steadily present at relatively high concentration in the plasma under normal dietary conditions. In the plasma, retinol binding protein binds this hydrophobic molecule and ensures receptor-mediated uptake by cells, where retinoid is metabolized to several active molecules including all-trans retinoic acid and 9-cis retinoic acid (9-cis RA) [154]. RXR is mainly bound and activated by 9-cis RA [155], and the activated RXR forms a heterodimer with LXR to induce expression of target genes including *ABCA1*. Also, active RXR can be heterodimerized with RAR, which is bound by all-trans retinoic acid and 9-cis RA [155], to induce target genes including *ABCA1*, *LXRα*, and *CYP27A1* [134].

The expression level of ABCA1 is also modulated at post-translational steps. It has been reported that unsaturated fatty acids [131] and increased cell unesterified cholesterol [156] promote the degradation of ABCA1 protein in macrophages without altering ABCA1 mRNA abundance, whereas apolipoproteins protect ABCA1 protein from degradation [157]. Yamauchi *et al.* [158] proposed that apoA-I-mediated sphingomyelin efflux activates protein kinase C (PKC), that then phosphorylates ABCA1 to prevent its degradation by calpain protease.

The activity of ABCA1 is modulated by several protein kinases. See *et al.* [159] reported that PKA-mediated phosphorylation of serine-1042 and -2054 residues in the nucleotide binding domains of ABCA1 occurs constitutively and is essential for optimum lipid-transport activity, without affecting apoA-I binding or ABCA1 protein stability. Tang *et al.* [160] reported that the interaction of apoA-I with ABCA1-expressing cells activates tyrosine kinase Janus kinase 2 (JAK2) by stimulating autophosphorylation of JAK2, which in turn phosphorylates unknown proteins to enhance apoA-I binding to cells and ABCA1-dependent lipid efflux, but without affecting ABCA1 protein content or ABCA1 phosphorylation. Roosbeek *et al.* [161] reported that protein kinase CK2 phosphorylates threonine-1242, threonine-1243, and serine-1255 residues downstream of the first nucleotide binding domain of ABCA1, and that mutations preventing protein kinase CK2 phosphorylation enhance apolipoprotein binding to cells and ABCA1-dependent lipid efflux.

1.3 Niemann-Pick type C (NPC) disease and ABCA1-dependent cholesterol efflux

NPC disease, caused by mutations in either the *NPC1* or *NPC2* genes, is a severe, inherited intracellular lipid trafficking disorder in which patients exhibit progressive neurodegeneration and hepatosplenomegaly [14, 30, 162]. Most cases (~95%) result from mutations in the *NPC1* gene and more than 170 mutations have been identified in NPC1 patients [162]. The NPC1 protein is a ubiquitously expressed, 1278 amino acid membrane glycoprotein that is primarily localized in late endosomes and travels to lysosomes and the *trans*-Golgi network [163, 164]. Structural analysis has predicted that NPC1 protein contains 13 transmembrane domains, three heavily glycosylated large loops, and a sterol-sensing domain [162] (Figure 1-9). Ohgami *et al.* [18] suggested that the sterol-sensing domain is required for direct binding to cholesterol. As mentioned in Section 1.1.3, the direct binding of cholesterol to the sterol-sensing domain in SCAP plays a key role in regulating the cholesterol biosynthetic pathway, supporting an important role for NPC1 in cholesterol metabolism.

In support of this, *NPC1* mutations are characterized by the accumulation of cholesterol, gangliosides and other glycosphingolipids in late endosomes/lysosomes [165, 166], and reintroduction of the *NPC1* gene in NPC1-deficient cells corrects the cholesterol trafficking defect [167]. It has not been answered yet whether the accumulation of cholesterol is a primary cause of NPC disease or the accumulation of other lipids, nor whether NPC disease is caused by excess lipid accumulation or impaired lipid trafficking. Double KO ($Npc1^{-/-}GalNAcT^{-/-}$) mice, which are deficient in biosynthesis of both NPC1 and complex gangliosides, exhibit the same life-span and neuropathology seen in $Npc1^{-/-}$ mice, even though the double KO mice did not accumulate several gangliosides that are accumulated in $Npc1^{-/-}$ mice [168]. This study suggests that the accumulation of gangliosides affected by GM2/GD2/GA2 synthase, encoded by the beta 1,4 N- acetylgalactosaminyltransferase gene (*GalNAcT*) [169], is not responsible for NPC disease. *Npc1*$^{-/-}$*GalNAcT*$^{-/-}$ mice showed marked reduction in the accumulation of UC in the brain, but not in the liver, compared with *Npc1*$^{-/-}$ mice [168, 170]. Cruz and Chang [171] reported that the cholesterol trafficking defect in NPC1 cells is not caused by ganglioside accumulation. This was based on their observation that the treatment of *N*-butyldeoxynojirimycin (NB-DNJ), an inhibitor of complex ganglioside biosynthesis, depleted GM3, GM2, GM1, and GD1a gangliosides in 25RA cells, a CHO cell line, and CT43 cells, NPC1-defective 25RA cells, however sterol biosynthesis and cholesterol esterification were not changed in these cells. Much more data are required to target a specific lipid species relevant to treating this disease.

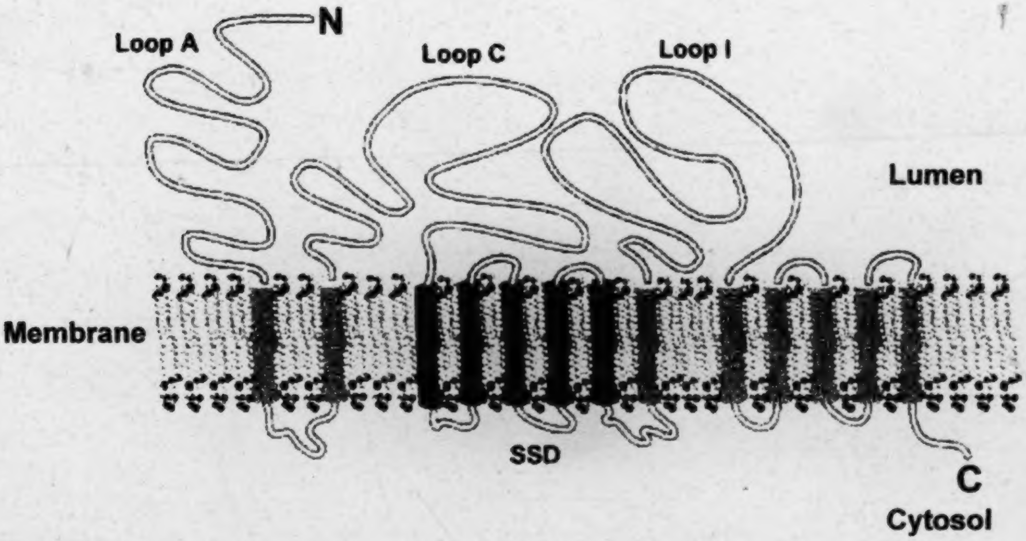


Figure 1-9. Current topological model of NPC1. The three large luminal loops (loop A, C, and I) have been predicted to be heavily glycosylated. Five of the transmembrane domains (transmembrane 3-7) have sequence homology to the sterol sensing domain (SSD). The figure was obtained from C. Scott and Y.A. Ioannou [162] and is presented here with permission.

The involvement of NPC1 in lipid trafficking from late endosomes/lysosomes to the *trans*-Golgi network and the plasma membrane has been proposed. Puri *et al.* [172] observed that a fluorescent lipid analogue (BODIPY-labeled lactosyl ceramide) was localized predominantly in the *trans*-Golgi network in normal cells, whereas most of the label was distributed throughout the cytoplasm in punctate vesicles characteristic of endosomes and lysosomes in NPC1 deficient cells. Millard *et al.* [173] reported that overexpression of NPC1 increases the transport of LDL-derived cholesterol to the plasma membrane.

Although the exact function of NPC1 protein has not yet been assigned, previous findings indicate that NPC1 plays an essential role in trafficking of late endosomal/lysosomal lipids to other cellular destinations. As depicted in Figure 1-5, LDL-derived cholesterol is metabolized through the endosomal/lysosomal system, and NPC1 deficiency sequesters LDL-derived cholesterol within the late endosomes/lysosomes, the biochemical hallmark of NPC disease. It has been reported that endogenously synthesized cholesterol is also accumulated within NPC1 compartment in NPC1-deficient cells [171].

The sequestration of cholesterol in late endosomes/lysosomes of NPC cells impairs key regulatory steps of cholesterol homeostasis that normally occur in response to excess cellular cholesterol, *i.e.*, down-regulation of HMG-CoA reductase and LDL receptor, and up-regulation of cholesterol esterification by ACAT [174-177]. The impaired regulation of cholesterol biosynthetic and influx pathways suggests that the ABCA1-dependent cholesterol efflux pathway may also be defective in NPC1 cells, since ABCA1 expression is strongly regulated by cell cholesterol content (as described in Section 1.2.5). In addition, the retroendocytosis model of ABCA1 function (Section 1.2.4.1) proposes internalized ABCA1/apoA-I mobilizes late endosomal/lysosomal cholesterol to the cell surface for secretion as HDL particles. This model suggests that the accumulation of cholesterol in late endosomes/lysosomes in NPC disease could be due to impaired ABCA1 function.

In this thesis, the expression of ABCA1 and its function on lipid efflux and HDL particle formation were studied in NPC1-defecient human fibroblasts. In conjunction with *in vitro* studies, HDL levels in NPC patients were also investigated.

1.4 Atherosclerotic smooth muscle cells and ABCA1-dependent cholesterol efflux

Atherosclerotic lesions (atheromas) are asymmetric focal thickenings of the innermost layer of the artery, the intima. Several cell types including blood-borne inflammatory and immune cells, endothelial cells, and smooth muscle cells (SMC) contribute to the development of atheroma. SMC represent the predominant cell type in the intima. A major role of intimal SMC is thought to be the production of collagen and other components of extracellular matrix, which form a fibrous intimal tissue distinct from the fibrous tissue formed by fibroblasts in usual wound repair [178, 179]. The fibrous intimal tissue is heavily infiltrated with circulating apoB100-containing lipoproteins such as LDL and VLDL. Extracellular matrix components such as proteoglycans bind to apoB100-containing lipoproteins, leading to their retention and modification [180, 181]. Intimal SMC, like intimal macrophages, take up the modified lipoproteins via scavenger and other receptors, accumulate large amounts of lipids, and become foamy appearing SMC, like macrophage foam cells, in the intima [182-186]. Over-accumulation of lipids and other toxic agents induces apoptotic and/or necrotic death of intimal SMC [187-189], which leads to discharge of the accumulated lipids and other cellular elements into the extracellular space, forming a lipid-rich necrotic core. As the necrotic core grows in the center of atheroma, the fibrous intimal tissue appears as a cap. As more intimal SMC die, the fibrous cap will become weakened and eventually degenerated, resulting in rupture of the atheroma. Although several species of lipids are accumulated in atheroma, the accumulation of CE is considered as a biochemical marker of the development of atherosclerosis. It is therefore pivotal to prevent over-accumulation of cholesterol in intimal SMC for the stabilization of atheromas.

Arterial SMC have been identified to be heterogeneous in morphology and biochemical properties in various species including human (reviewed in [190]). Arterial SMC have been categorized into at least two distinct subtypes, spindle-shaped SMC and epithelioid-shaped SMC (Table 1-1). Features of another subtype, rhomboid-shaped SMC, were similar to epithelioid-shaped SMC and distinct features of these two SMC subtypes included greater proliferation, faster migration, and poor differentiation as defined by the expression levels of cytoskeletal and contractile proteins, when compared to spindle-shaped SMC (Table 1-1).

Table 1-1. Biological and biochemical features of SMC subpopulations.

Species	Rat		Cow			Pig		Human	
Phenotype	Spindle	Epithelioid	Spindle	Rhomboid	Epithelioid	Spindle	Rhomboid	Spindle	Epithelioid
Autonomous growth	No	Yes	No	Yes	Yes	No	No	No	No
Migratory activity	Low	High	ND	ND	ND	Low	High	Low*	High*
Differentiation features									
α-SM actin	++	+	++	+/−	+	+++	+	+++	++
Desmin	+/−	−	ND	ND	ND	+	+/−	ND	ND
SMMHC	+	+/−	++	−	−	++	+	+++	++
Smoothelin	ND	ND	ND	ND	ND	+	+/−	ND	ND
SM22α	++	ND	++	+/−	+	ND	ND	ND	ND

* Under platelet-derived growth factor. SM, smooth muscle; SMMHC, smooth muscle myosin heavy chain; ND, not determined. The table was obtained from H. Hao *et al.* [190] and is presented here with permission.

It has been reported that spindle-shaped contractile SMC are typical of the normal artery medial layer, whereas epithelioid-shaped synthetic SMC are predominant in atherosclerotic artery intima [190-192]. Although the change in the proportion of arterial SMC in response to atherosclerotic injury might be a part of vascular adaptation to meet altered functional demands and to maintain the structural integrity of injured arteries, the accumulation of CE in atherosclerotic intimal SMC, but not in normal SMC [183, 193] strongly suggests that altered cholesterol metabolism in intimal SMC may be a key contributing factor in the progression of atherosclerosis.

As mentioned in Section 1.1.4 of cholesterol homeostasis, excess cholesterol in cells is removed by cholesterol efflux pathways, but cholesterol efflux from intimal SMC has not been studied in detail. In this thesis, to examine whether ABCA1-dependent cholesterol efflux from intimal SMC is different from normal medial SMC, epithelioid-shaped synthetic SMC and spindle-shaped contractile SMC lines have been utilized as models of the major intimal and normal arterial SMC subtypes, respectively.

1.5 Thesis objectives

Dynamic intracellular cholesterol trafficking allows cells to sense their cholesterol content and therefore to regulate cholesterol homeostasis mechanisms including biosynthesis, influx, and efflux of cellular cholesterol. Dysfunction of the NPC1 protein results in the sequestration of cholesterol in late endosomes and lysosomes. Previous studies have demonstrated that NPC1 deficiency impaired cholesterol biosynthesis and influx regulation, however the effect of NPC1 deficiency on the cholesterol efflux mechanism has not been studied in detail. The lipid transporter ABCA1, which mediates the critical step of lipid efflux to lipid-free/poor apoA-I to form HDL particles, is also regulated by cell cholesterol content. It is therefore hypothesized that NPC1 deficiency impairs the regulation of ABCA1 and therefore the ABCA1-dependent cholesterol efflux mechanism.

In Chapter 3, ABCA1 expression levels and ABCA1-dependent lipid efflux were examined in normal, NPC1$^{+/-}$, and NPC1$^{-/-}$ human fibroblasts. To confirm results obtained from cell studies *in vivo*, plasma lipid profiles of NPC patients were investigated. To test whether impaired ABCA1 regulation in NPC1$^{-/-}$ cells is corrected by exogenously added ABCA1 inducers, cells were treated with a synthetic ABCA1 inducer and the effects on ABCA1 expression, ABCA1-dependent lipid efflux, and HDL particle formation were determined.

A biochemical marker of atherosclerotic lesions is accumulation of CE in the intimal layer of atheromas. Cloning studies have demonstrated that arterial SMC are heterogeneous in morphology and biological functions. Normal arteries are mainly composed of spindle-shaped contractile SMC, whereas the major SMC subtype in atherosclerotic lesions is epithelioid-shaped synthetic SMC. The accumulation of CE in atherosclerotic arteries but not in normal arteries suggests that epithelioid-shaped synthetic SMC may have impaired ABCA1-dependent cholesterol efflux. To test this possibility, the following studies were performed and are divided into two Chapters:

In Chapter 4, ABCA1 expression levels and ABCA1-dependent lipid efflux were examined in three epithelioid-shaped synthetic and three spindle-shaped contractile SMC lines.

In Chapter 5, to test whether an increase in ABCA1 expression can correct impaired ABCA1-dependent lipid efflux from epithelioid-shaped synthetic SMC, cells were treated with a synthetic ABCA1 inducer or transfected with ABCA1 construct, and the effects on ABCA1-dependent lipid efflux and apoA-I binding were determined.

In Chapter 6, the research is summarized and proposed future research presented.

CHAPTER 2:

General Methodology

The following methods were used in the research studies presented in the next three Chapters, and are therefore provided only once here. Additional methods unique to each study are provided in the individual Chapters.

2.1 Materials

Cholesterol, phosphatidylcholine (PC), sphingomyelin (SM), and essentially fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Oakville, ON. [1,2-^{3}H]Cholesterol, [*methyl*-^{3}H]choline chloride, and [*cholesteryl*-1,2,6,7-^{3}H]cholesteryl linoleate were purchased from NEN Life Science Products, Boston, MA, and (*RS*)-[2-^{14}C]mevalonic acid lactone and Na^{125}I were from Amersham Biosciences, Baie d'Urfé, QC. The organic solvents used in this study were purchased from Fisher Scientific, Ottawa, ON.

Dulbecco's modified Eagle's medium (DMEM) was purchased from BioWhittaker, Walkersville, MD, and lipoprotein-deficient serum and fetal bovine serum (FBS) from Hyclone, Logan, UT.

Nitrocellulose membranes, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents, and pre-stained protein molecular mass markers were purchased from Bio-Rad Laboratories, Mississauga, ON.

2.2 Preparation of lipoproteins and apoA-I

HDL (d = 1.063–1.21 g/ml) and LDL (d = 1.019–1.063) were isolated by standard ultracentrifugation techniques from the pooled plasma of healthy volunteers [194]. HDL fractions were subjected to heparin-Sepharose affinity chromatography to remove apoE- and apoB-containing particles [195]. The whole protein fraction of HDL was obtained by delipidating HDL and purified apoA-I obtained using DEAE-cellulose chromatography as described [196]. Purified apoA-I was lyophilized and stored at -80 °C. ApoA-I was dissolved in 66 mM KH_2PO_4 containing 0.1 mM diethylenetriamine pentaacetic acid (pH 8.0) at a concentration of 1 mg/ml before use. LDL was labeled with [1,2,6,7-^{3}H]cholesteryl linoleate by the method of Sattler and Stocker [197] to a specific activity of ~14 cpm/ng LDL protein. For apoA-I binding assays, apoA-I was iodinated with ^{125}I by IODO-GEN (Pierce, Rockford, IL) to a specific activity of ~ 860 cpm/ng apoA-I.

2.3 Cell culture

All cell lines were cultured in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin in humidified 95% air and 5% CO_2 at 37°C unless specifically indicated.

To load cells with non-lipoprotein cholesterol, confluent cells were rinsed twice with phosphate-buffered saline (PBS) containing 1 mg/ml BSA (PBS/BSA) and incubated for 24 h in DMEM containing 2 mg/ml BSA with 30 µg/ml cholesterol added from a 10 mg/ml stock in ethanol. To allow equilibration of added cholesterol, cells were rinsed twice with PBS/BSA and incubated for an additional 24 h in DMEM containing 1 mg/ml BSA (DMEM/BSA).

2.4 Labeling of cellular cholesterol pools and choline-containing phospholipids

To radiolabel LDL-derived cellular cholesterol pools, cells were incubated in DMEM containing 10% lipoprotein-deficient serum during the last 40% of growth to confluence to up-regulate LDL receptor expression and then incubated for 24 h with 50 µg/ml [^{3}H]cholesteryl linoleate-labeled LDL (protein). Cells were then rinsed 3 times with PBS/BSA prior to addition of apoA-I.

To radiolabel non-LDL-derived cellular cholesterol pools, rapidly growing cells were labeled during the last 40% of growth to confluence by addition of 0.2 µCi/ml [^{3}H]cholesterol in DMEM containing 10% FBS [198]. Radiolabeled cells were loaded with non-lipoprotein cholesterol prior to addition of apoA-I.

To label more selectively plasma membrane cholesterol pools, cholesterol-loaded and equilibrated cells were incubated for 2 h with DMEM/BSA containing 0.2 µCi/ml [^{3}H]cholesterol followed by extensive rinse prior to addition of apoA-I [199].

To label newly synthesized cholesterol, rapidly growing cells were incubated with DMEM/BSA containing 0.5 µCi/ml [^{14}C]mevalonic acid lactone during the last 40% of growth to confluence. Cells were then rinsed 3 times with PBS/BSA, equilibrated 24 h in DMEM/BSA, and rinsed 3 times with PBS/BSA prior to addition of apoA-I.

Choline-containing phospholipids were labeled in cholesterol-loaded cells by addition of 3 µCi/ml [^{3}H]choline chloride to the DMEM/BSA medium during the 24 h equilibration period. Cells were rinsed 5 times with PBS/BSA prior to addition of apoA-I [119].

2.5 Cholesterol and phospholipid efflux

After the desired labeling protocol, cells were incubated for 1–48 h in DMEM/BSA containing 0-10 µg/ml apoA-I. At the end of the indicated incubation periods, cell layers were rinsed twice with iced PBS/BSA and twice with iced PBS. Cells were stored at –20 °C until lipid extraction.

Efflux media were collected and centrifuged at 3,000 rpm (2,045 x g) for 10 min to remove cell debris. The supernatant efflux media were then either counted directly for determination of [^{3}H]cholesterol or extracted with chloroform/methanol (2:1, v/v) as described [200] for separation of [^{3}H]choline-containing phospholipids. Cellular lipids were extracted with hexane/isopropanol (3:2, v/v) as described [201].

To separate cellular sterol species, cellular extracts were dried under air, re-dissolved in a small volume of chloroform, and applied to silica gel G thin-layer chromatography plates (Whatmann Inc., Florham Park, NJ). The plates were developed in hexane/diethyl ether/acetic acid (130:40:1.5, v/v/v). To separate choline-containing phospholipids, medium and cellular extracts were dried under air, re-dissolved in a small volume of chloroform and applied to silica gel H thin-layer chromatography plates (Fisher Scientific, Ottawa, ON). The plates were developed in chloroform/methanol/acetic acid/water (100:60:16:8, v/v/v/v). Lipid spots were visualized by exposure of thin-layer chromatography plates to iodine vapor and identified by comparing their migration with standards. Target spots were taken and dissolved in 4.5 ml of EcoLite liquid scintillation cocktail (ICN Biomedicals, Irvine, CA). The radioactivity associated with each lipid was determined by a Beckman LS 6000TA counter. Cell proteins were determined by the Lowry assay using BSA as standard [202].

2.6 Sterol mass analysis by gas chromatography

Incubation condition of cells for sterol mass analysis is described in the relevant Chapters. At the end of the incubation, media and cells were collected. Phospholipids from media or cell homogenates were digested by phospholipase C to remove the polar head groups, and total lipids were extracted in the presence of tridecanoin as the internal standard. Samples were derivatized with Sylon BFT (Supelco, Bellefonte, PA) and analyzed by gas chromatography (Agilent Technologies, 6890 Series equipped with a Zebron capillary column (ZB-5, 15 m x 0.32 mm x 0.25 µm) and connected to a flame ionization detector; Palo Alto, CA). The oven temperature was raised from 170 to 290 °C at 20 °C/min, and then to 340°C at 10 °C/min where the temperature was kept for 24 min. Helium (He) was used as the carrier gas. The gas chromatography was operated in constant flow mode with a flow rate of 4.5 ml He/min. The injector was operated in the split mode and was kept at 325 °C, and the detector was kept at 350 °C. Separation of sterols was identified by comparing their retention times with standards, and calculation of sterol mass in samples was based on the internal standard.

2.7 Protein electrophoresis and immunoblot analysis

In preparation of immunoblotting of proteins, crude cellular membrane proteins or whole cell lysates were processed as described in individual Chapters. Protein concentrations were determined by the Lowry assay using BSA as standard [202]. Proteins in the samples were resolved by SDS-PAGE by the method of Laemmli [203] and transferred onto nitrocellulose membranes by electroblotting for 16 h at 4 °C. Membranes were blocked 1 h at room temperature with 5% skimmed milk power in Tris-buffered saline (TBS; 25 mM Tris, 137 mM NaCl, pH 7.5) containing 0.1% Tween 20 (TBS/T), rinsed three times with TBS/T, and incubated for 1 h at room temperature or 16 h at 4 °C with primary antibodies diluted in TBS/T containing 1% skimmed milk powder or 5% BSA (essentially protease free) at ratios indicated in individual Chapters. Following the incubation, membranes were rinsed five times with TBS/T and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Typically secondary antibodies were diluted in TBS/T containing 1% skimmed milk powder at ratios of 1:5,000 or 1:10,000 antibody to TBS/T. Membranes were extensively rinsed with TBS/T before chemiluminescent detection using the enhanced chemiluminescence assay system (Amersham Biosciences, Baie d'Urfé, QC).

2.8 Statistical analysis

Statistics were performed using Prism software (GraphPad, San Diego, CA). Results are presented as the mean ± S.D. Comparison of 2 samples was performed by a two-tailed Student *t* test and for 3 or more samples ANOVA was performed with either Dunnett's multiple test for comparison of each group vs. control, or Newman-Keuls multiple test for comparison of all groups with each other.

CHAPTER 3:

Impaired ABCA1-dependent Lipid Efflux and Hypoalphalipoproteinemia in Human Niemann-Pick type C Disease

3.1 Introduction

3.1.1 Niemann-Pick type C disease and cholesterol accumulation

Niemann-Pick type C (NPC) disease is a neurodegenerative disorder characterized by a variable phenotype but that frequently leads to premature death in childhood or adolescence [204]. Biochemically the disorder is characterized by impaired intracellular lipid trafficking, with accumulation of unesterified cholesterol and glycosphingolipids in late endosomes/lysosomes [175, 205]. Recent studies [163, 206] have indicated the NPC1 protein resides in a unique late endosomal compartment that becomes enriched with cholesterol in cells with dysfunctional NPC1. Although the exact function of the NPC1 protein remains unknown, it is believed to facilitate the transport of lipids, particularly cholesterol, from late/endosomes lysosomes to the Golgi apparatus, endoplasmic reticulum, and plasma membrane [207-209]. Impaired cholesterol trafficking in NPC1-deficient cells results in blunted down-regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) and LDL receptor activity, and a defect in the delivery of unesterified cholesterol to the ER for esterification by acyl-CoA:cholesterol acyltransferase (ACAT) [174, 176, 177].

3.1.2 ATP-binding cassette transporter A1 (ABCA1)-dependent lipid efflux and NPC1 deficiency

The membrane protein required for lipidation of apolipoprotein A-I (apoA-I), ABCA1, is up-regulated in response to increased cell cholesterol, leading to high density lipoprotein (HDL) particle formation and assisting with the maintenance of cell cholesterol homeostasis (reviewed in Ref. [210]). ABCA1 mediates the rate-limiting step of HDL particle formation and is thought to function by transferring cellular phospholipids and/or cholesterol to lipid-free/poor apoA-I [210]. Mutations in *ABCA1* result in a failure of lipidation of apoA-I [88], increased intracellular cholesterol, and extremely low HDL levels in the hypoalphalipoproteinemic syndrome Tangier disease [211]. ABCA1 expression is normally upregulated by increasing cell cholesterol and oxysterol content, as seen in arterial wall macrophages in atherosclerosis, through oxysterol-dependent activation of the nuclear transcription factor liver X receptor (LXR) [136, 212]. Although HDL levels in human subjects with NPC disease had not been reported, the failure to regulate appropriately other cholesterol metabolic genes in NPC disease predicts ABCA1 regulation and function would also be impaired in this disorder, resulting in decreased HDL particle formation.

3.1.3 Study objectives

To test the hypothesis that ABCA1 regulation and function are impaired in NPC1-deficient cells, apoA-I-mediated efflux of phospholipids and cholesterol from distinct cellular pools, cellular binding of apoA-I, and regulation of *ABCA1* expression were examined in normal (*NPC1*$^{+/+}$), *NPC1*$^{+/-}$, and *NPC1*$^{-/-}$ human fibroblasts. To see whether results obtained from cell studies correlate with HDL metabolism in patients, the plasma lipid profiles of NPC patients were also investigated.

3.2 Methods

3.2.1 Cell culture

Normal human skin fibroblasts (*NPC1*$^{+/+}$, CRL-2076) were purchased from the American Type Culture Collection (Manassas, VA). *NPC1* heterozygous human fibroblasts (*NPC1*$^{+/-}$) containing the L1213V mutation were generously provided by Dr. David Byers (Dalhousie University) [213]. *NPC1* compound heterozygote human fibroblasts containing the most prevalent *NPC1* mutation [214], I1061T, and the P237S mutation (*NPC1*$^{-/-}$, GM3123) were purchased from the Human Mutant Cell Repository (Camden, NJ). The *NPC1*$^{-/-}$ cells are from an affected child and have been shown previously to have a severe defect in cholesterol esterification [215]. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

3.2.2 Filipin staining

Cells were grown to confluence on glass coverslips in DMEM containing 10% FBS, incubated for 24 h in DMEM containing 1 mg/ml essentially fatty acid-free bovine serum albumin (DMEM/BSA) to allow equilibration of cholesterol, rinsed three times with phosphate-buffered saline (PBS), and fixed in 3% paraformaldehyde for 15 min at room temperature in PBS. The cells were rinsed three times with PBS, incubated with 1.5 mg glycine/ml in PBS for 10 min at room temperature, rinsed three times with PBS, and stained with 50 µg/ml filipin in PBS for 1 h at room temperature [216]. The cells were rinsed three times with PBS and mounted in Prolong Antifade mounting medium (Molecular Probes, Burlington, ON). Filipin fluorescence was detected under a Leica DM IRE2 digital microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Fluotar ×63/0.70 objective and a Leica ebq100 fluorescence lamp.

3.2.3 Cholesterol efflux assay

Cells were seeded on 16-mm wells. Four distinct cellular cholesterol pools (LDL-derived, non-lipoprotein-derived, plasma membrane, and newly synthesized cholesterol pools) were radiolabeled, and efflux assays were performed as described in Sections 2.3 – 2.5.

3.2.4 Phospholipid efflux assay

Cells were seeded on 35-mm wells, choline-containing phospholipids were radiolabeled, and efflux assays were performed as described in Sections 2.3 – 2.5.

3.2.5 Sterol mass analysis by gas chromatography

Cells grown to ~60% confluence in DMEM containing 10% FBS on 60-mm dishes were incubated in DMEM containing 10% lipoprotein-deficient serum during the last 40% growth to up-regulate LDL receptor expression. Confluent cells were rinsed twice with PBS/BSA and incubated for 24 h with 50 μg/ml LDL (protein). The cells were rinsed twice with PBS/BSA and equilibrated for 24 h in DMEM/BSA with or without 5 μM TO-901317. The cells were then rinsed twice with PBS/BSA and incubated for 24 h with 10 μg/ml apoA-I in the absence or presence of 5 μM TO-901317. At the end of the incubation, media and cells were collected for sterol mass analyses as described in Section 2.6.

3.2.6 Cellular binding of apoA-I

The binding of apoA-1 to cells was determined as described previously [217]. Non-cholesterol-loaded cells or cells loaded with non-lipoprotein cholesterol in 35-mm wells were incubated for 2 h at 0 °C in DMEM/BSA containing 25 mM HEPES and increasing concentrations of ^{125}I-apoA-I. The medium was then removed and cells were rinsed 5 times with iced PBS/BSA and twice with iced PBS. Cell layers were dissolved in 0.1 N NaOH, and aliquots were taken for quantitation of radioactivity and protein.

3.2.7 Reverse transcriptase-PCR analysis of ABCA1 mRNA

Total RNA was isolated from cells by guanidine isothiocyanate/phenol/ chloroform extraction [218]. The concentration of RNA was measured spectrophotometrically at a wavelength of 260 nm, and 2 µg of RNA was treated with DNase I (Invitrogen, Burlington, ON) following the manufacturer's guidelines. First strand cDNA synthesis was performed using 500 nM of oligo(dT) primer and Superscript™ RNase H (Invitrogen). Each reaction mixture contained 100 units of Superscript™ enzyme, 1x first strand buffer (50 mM Tris-HCl, pH 8.0), 0.5 µM dNTP mix, 0.01 M dithiothreitol, 0.05 µg/µl BSA, and 2 units of RNase inhibitor (Invitrogen). The mixtures were incubated at 45 °C for 90 min followed by incubation at 95 °C for 3 min (Whatman Biometra T-gradient thermocycler) and then put promptly on ice. Amplification of *ABCA1* and cyclophilin mRNAs was performed in tandem to ensure equal amounts of starting cDNA for each sample. Diethyl pyrocarbonate-treated water, 1x PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 1.5 mM $MgCl_2$, 0.1 mM dNTPs, and cDNA were added to 200 µl of thin walled PCR tubes and mixed, and one-half volume was transferred to another PCR tube. One unit of *Taq* DNA polymerase (Invitrogen) and 2 µl of 10 µM forward and reverse primers (ABCA1 or cyclophilin) were added to complete the reaction mixture. *ABCA1* amplification was performed by initially denaturing DNA at 95 °C for 3 min. Thereafter, denaturing was at 95 °C for 75 s, annealing at 54.6 °C for 75 s, and extension at 72 °C for 55 s for a total of 31 cycles with a final extension period of 5 min. Human cyclophilin amplification was performed using similar conditions except the annealing temperature was 48 °C with a total of 33 cycles. PCR products were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light. The primers used were as follows: human ABCA1, 5'-GAC ATC CTG AAG CCA ATC CTG (forward), 5'-CCT TGT GGC TGG AGT GTC AGG T (reverse); human cyclophilin, 5'-ACC CAA AGG GAA CTG CAG CGA GAG C (forward), 5'-CCG CGT CTC CTT TGA GCT GTT TGC AG (reverse).

3.2.8 Northern blot analysis of ABCA1 mRNA

Total RNA was isolated from cells as described [218]. Seven micrograms of RNA were electrophoresed on a 1% agarose gel containing 5% formaldehyde in 20 mM 4-morpholinepropanesulfonic acid (MOPS), 5 mM sodium acetate, 1 mM ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) buffer, pH 7.0, and transferred onto a nylon membrane (Amersham Biosciences) by capillary transfer. The probe for *ABCA1* was obtained by purifying PCR products described above using a gel extraction kit (Qiagen, Mississauga, ON) and then radiolabeled by the random priming method with [α-^{32}P]dCTP (Invitrogen). After cross-linking with UV light (Stratalinker model 1800, Stratagene), the membranes were hybridized with ^{32}P-labeled probes. The hybridization signal was detected by autoradiography.

3.2.9 Western blot analysis of ABCA1 protein

To prepare crude cellular membrane proteins, cells were homogenized on ice in 50 mM Tris-HCl buffer, pH 7.4, containing protease inhibitors and 2 mM EDTA. The nuclear fraction was removed by centrifugation for 10 min at 700 rpm (40 x g), and the supernatant was subsequently centrifuged for 20 min at 14,000 rpm (16,000 x g). The pellet, resuspended in 0.45 M urea containing 0.1% Triton X-100 and 0.05% dithiothreitol, was used as crude cellular membrane proteins [219]. To prepare total tissue proteins, dissected tissues were homogenized on ice in radio-immunoprecipitation assay (RIPA) buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 4 mM EDTA] containing complete protease inhibitors, and then lysed in the RIPA buffer for 30 min on ice [156]. The homogenate was centrifuged for 20 min at 14,000 rpm (16,000 x g), and the supernatant was used as total tissue proteins. Thirty micrograms of crude cellular membrane proteins or 100 μg of total tissue proteins were separated by 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. Immunoblotting was performed as described in Section 2.7 using a polyclonal rabbit anti-human ABCA1 antibody (1:500 dilution) (a kind gift of Dr. Shinji Yokoyama, Nagoya City University [219]) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000, Sigma-Aldrich, Oakville, ON).

3.2.10 Lipid profiles of NPC patients

Fasting lipid profiles for 21 NPC1-deficient subjects (10 male and 11 female, age ranges 3–42) and 31 NPC heterozygous parents (15 males and 16 females) were obtained from routine clinical laboratory analyses with the assistance of the Ara Parseghian Medical Research Foundation (Tucson, AZ).

3.2.11 Two-dimensional gel electrophoresis of HDL particles

To characterize apoA-I-containing particles generated by *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ human skin fibroblasts, cell-conditioned media in 35 mm dishes were centrifuged at 2000 rpm (912 x g) for 5 min at 4°C to pellet cells and the supernatant was concentrated 10-fold by ultrafiltration (Amicon Ultra-4, MWCO 10000 from Millipore, Cambridge, ON). Media samples were kept on ice and used the same day or frozen at -20°C. Freezing at -20°C and thawing did not affect HDL particle size distribution or abundance on two-dimensional gel electrophoresis. HDL particles in equivalent volumes of concentrated apoA-I-conditioned media were separated according to the method of Castro and Fielding [220] except that in the second dimension, voltage was increased from 100V for 19 h to 125V for 24 h to increase the separation of the α-migrating HDL species. Briefly, 20 µl samples were separated in the first dimension by 0.75% agarose gel in 50 mM barbital buffer, pH 8.6, at 200 V for 5.5 h at 5°C. Electrophoresis in the second dimension was performed with a 2-23% polyacrylamide concave gradient gel at 125 V for 24 h at 5°C in 0.025 M Tris, 0.192 M glycine buffer, pH 8.3. High molecular weight protein standards (7.1 to 17.0 nm from Amersham Pharmacia Biotech, Baie d'Urfé, QC) were run on each gel. Following electrophoresis, samples were electrotransferred (30 V, 24 h, 4° C) onto nitrocellulose membranes (Trans-Blot from Bio-Rad Laboratories, Mississauga, ON). To locate size markers, the nitrocellulose membranes were stained with Ponceau S and the position of each protein marked. Membranes were blocked by 1 h incubation in Tris-buffered saline containing 1% Tween 20 (TBS/T) and 10% skimmed milk at room temperature. ApoA-I-containing particles were detected by blotting the membranes with rabbit polyclonal anti-human apoA-I antibody (Calbiochem, Mississauga, ON) in TBS/T containing 1% skimmed milk for 1 h at room temperature, and then with [^{125}I]-labeled donkey-anti-rabbit antibody (Amersham Biosciences, Baie d'Urfé, QC) [221]. The specific activity of the secondary antibody was 4.8 X 10^{6} cpm/mg. Membranes were incubated for 3 h in 80 ml TBS/T containing 1% skimmed milk and 2.6 µg antibody, followed by three washes of 5 min each in TBS/T before autoradiography.

3.3 Results

3.3.1 Human NPC1$^{-/-}$ fibroblasts accumulate unesterified cholesterol

To confirm whether human *NPC1*-deficient fibroblasts used in this study accumulated unesterified cholesterol, cells were grown to confluence in DMEM containing 10% FBS and were stained with filipin. Filipin emits fluorescence after binding to unesterified cholesterol. *NPC1*$^{-/-}$ fibroblasts showed much brighter fluorescence compared with *NPC1*$^{+/-}$ and *NPC1*$^{+/+}$ fibroblasts (Figure 3-1), indicating that this *NPC1*$^{-/-}$ cell line accumulates unesterified cholesterol.

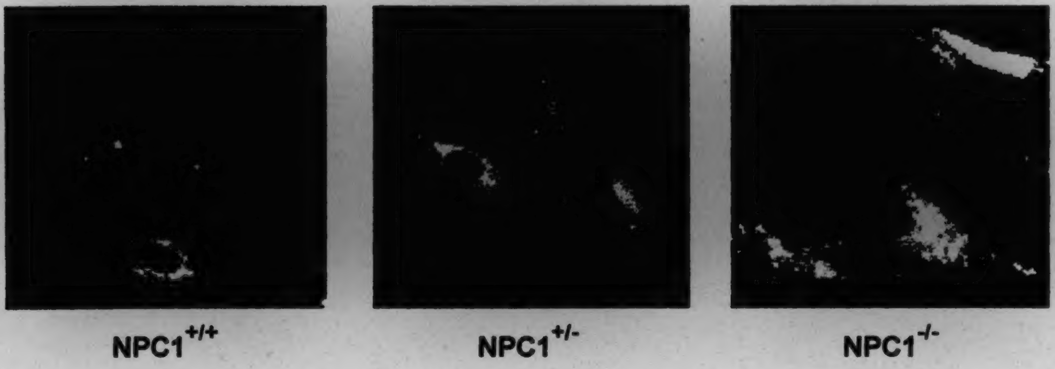


Figure 3-1. Accumulation of unesterified cholesterol in human NPC1-deficient fibroblasts. Human *NPC1*$^{+/+}$, *NPC1*$^{+/-}$, and *NPC1*$^{-/-}$ fibroblasts were grown to confluence in DMEM containing 10% FBS, incubated for 24 h in DMEM/BSA, and stained with filipin for 1 h at room temperature. Filipin fluorescence was visualized by fluorescent microscopy.

3.3.2 ApoA-I-mediated efflux of LDL-derived cholesterol is impaired in human $NPC1^{-/-}$ fibroblasts

Impaired trafficking of LDL-derived cholesterol in fibroblasts or lymphocytes is a biochemical hallmark of NPC disease [204]. To assess the removal of LDL-derived cholesterol by apoA-I in human NPC1-deficient fibroblasts, cells were grown to confluence in lipoprotein-deficient serum. Cells were then labeled with [^{3}H]cholesteryl linoleate-labeled LDL for 24 h prior to incubation with apoA-I. Incorporation of LDL-derived [^{3}H]cholesterol was approximately 3 times higher in $NPC1^{-/-}$ than in $NPC1^{+/+}$ or $NPC1^{+/-}$ cells (see Figure 3-2 legend), consistent with accumulation of cholesterol in late endosomes/lysosomes and a failure to down-regulate LDL receptor activity in $NPC1^{-/-}$ cells [176, 177]. Incubation of cells with 10 μg/ml apoA-I for 48 h resulted in efflux of 13–14% of LDL-derived [^{3}H]cholesterol to the medium from $NPC1^{+/+}$ cells (Figure 3-2A). $NPC1^{+/-}$ cells showed a slightly decreased ability to release LDL-derived cholesterol to apoA-I, whereas $NPC1^{-/-}$ cells showed markedly diminished efflux (only 2% above basal levels of efflux to albumin alone) to apoA-I compared with both these other cell lines. Removal of radiolabeled cellular cholesterol to the medium was accompanied by a marked decrease in radiolabeled cellular cholesteryl ester (CE) in $NPC^{+/+}$ and $NPC^{+/-}$ cells (Figure 3-2B). $NPC^{-/-}$ cells showed a sharper decline in cellular CE levels and a simultaneous accumulation of [^{3}H]cholesterol (Figure 3-2, B and C), consistent with normal rates of CE hydrolysis but failure to re-esterify cholesterol in the endoplasmic reticulum in $NPC^{-/-}$ cells [204].

3.3.3 ApoA-I-mediated efflux of total cell, plasma membrane, and newly synthesized cholesterol is impaired in human $NPC1^{-/-}$ fibroblasts

Accumulation of LDL-derived cholesterol in late endosomes/lysosomes in NPC1-deficient fibroblasts suggests that these compartments are the main site of NPC1 protein function [164, 206]. To investigate whether apoA-I-mediated efflux of cholesterol derived from non-lipoprotein sources is also impaired in human NPC1-deficient fibroblasts, cells were incubated with [^{3}H]cholesterol during the

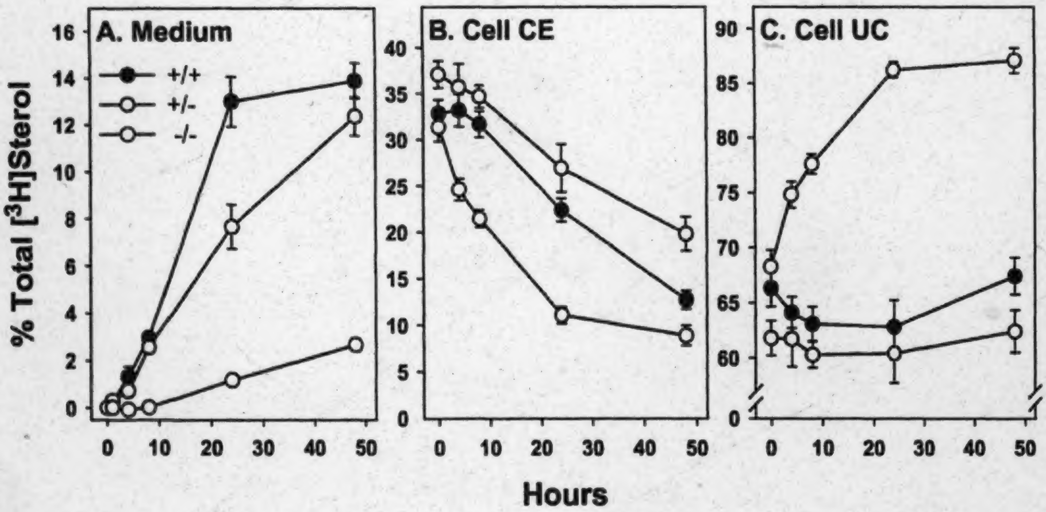


Figure 3-2. ApoA-I-mediated efflux of LDL-derived cholesterol from human NPC1-deficient fibroblasts. *NPC1*$^{+/+}$ (*black symbols*), *NPC1*$^{+/-}$ (*gray symbols*), and *NPC1*$^{-/-}$ (*white symbols*) fibroblasts incubated in lipoprotein-deficient serum were incubated for 24 h with 50 µg/ml [^{3}H]cholesteryl linoleate-labeled LDL and then with 10 µg/ml apoA-I for 1–48 h. At the indicated times the medium was removed, and cell cholesteryl ester (*CE*) and unesterified cholesterol (*UC*) in cells and media were analyzed for [^{3}H]cholesterol. Results are expressed as percent of total cell plus medium [^{3}H]sterol in the medium (*A*), cell CE (*B*), and cell UC (*C*) following subtraction of efflux to medium containing 1 mg/ml BSA alone. Cell [^{3}H]cholesterol immediately prior to addition of apoA-I was 94 ± 5, 115 ± 14, and 346 ± 19 x 10^3 dpm/mg cell protein for *NPC1*$^{+/+}$, *NPC1*$^{+/-}$, and *NPC1*$^{-/-}$ cells, respectively. Values are the mean ± S.D. of quadruplicate determinations and are representative of two experiments with similar results. *A*, values for *NPC1*$^{-/-}$ cells at $\geq$ 4 h and for *NPC1*$^{+/-}$ cells at 24 h are lower than *NPC1*$^{+/+}$ cells. *B*, values for *NPC1*$^{-/-}$ cells are lower than *NPC1*$^{+/+}$ cells at $\geq$ 4 h. *C*, values for *NPC1*$^{-/-}$ cells are greater than *NPC1*$^{+/+}$ cells at $\geq$ 4 h. For all significant differences, $p \leq 0.05$. The figure was published in Choi H.Y. *et al.* (2003) *J Biol Chem.* 278: 32569-32577.

last 40% of growth to label all cellular cholesterol pools. In other experiments, cells were pulse-labeled with [^{3}H]cholesterol for 2 h after confluence to label more specifically plasma membrane cholesterol [198]. It has been previously reported that a 2 h pulse of cholesterol-loaded normal human fibroblasts with [^{3}H]cholesterol results in less than 2% of labeled cholesterol being incorporated into cholesteryl esters [198]. Although cholesterol may be internalized without being esterified, we used this method to label more specifically the plasma membrane cholesterol pool. Cells were also incubated with [^{14}C]mevalonate lactone to label newly synthesized cholesterol. Consistent with the known defect in esterification of non-lipoprotein cholesterol, as well as LDL-derived cholesterol, in *NPC1*$^{-/-}$ cells [174], these cells esterified only 7.5 ± 1.8% of total cell [^{3}H]cholesterol delivered to cells during growth, compared with 31.4 ± 2.2 and 31.1 ± 1.3% in *NPC1*$^{+/+}$ and *NPC1*$^{+/-}$ cells, respectively. As shown in Figure 3-3, efflux of cholesterol to apoA-I from cells labeled by each of these methods was diminished from *NPC1*$^{-/-}$ fibroblasts compared with *NPC1*$^{+/+}$ cells. Diminished efflux from *NPC1*$^{-/-}$ cells occurred despite increased levels of [^{3}H]cholesterol and [^{14}C]mevalonate lactone incorporation by these cells during growth (see Figure 3-3 legend). Despite higher incorporation of [^{3}H]cholesterol during the pulse-labeling protocol, *NPC1*$^{+/-}$ cells showed intermediate levels of efflux of this pool of cholesterol to apoA-I compared with *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ cells (Figure 3-3B) and similarly intermediate levels of efflux of total cell (Figure 3-3A) and newly synthesized [^{3}H]cholesterol (Figure 3-3C). These results indicate that cholesterol efflux to apoA-I from *NPC1*$^{-/-}$ cells is diminished regardless of the pool of cellular cholesterol labeled.

3.3.4 ApoA-I-mediated removal of choline-containing phospholipids is defective in human NPC1-deficient fibroblasts

The ability of apoA-I to act as a cholesterol acceptor is thought to be dependent upon apoA-I being first or simultaneously phospholipidated in a process that requires ABCA1 [210]. [^{3}H]Choline-labeled *NPC1*$^{-/-}$ cells showed a diminished ability to mobilize both PC and SM to apoA-I (Figure 3-4). *NPC1*$^{+/-}$

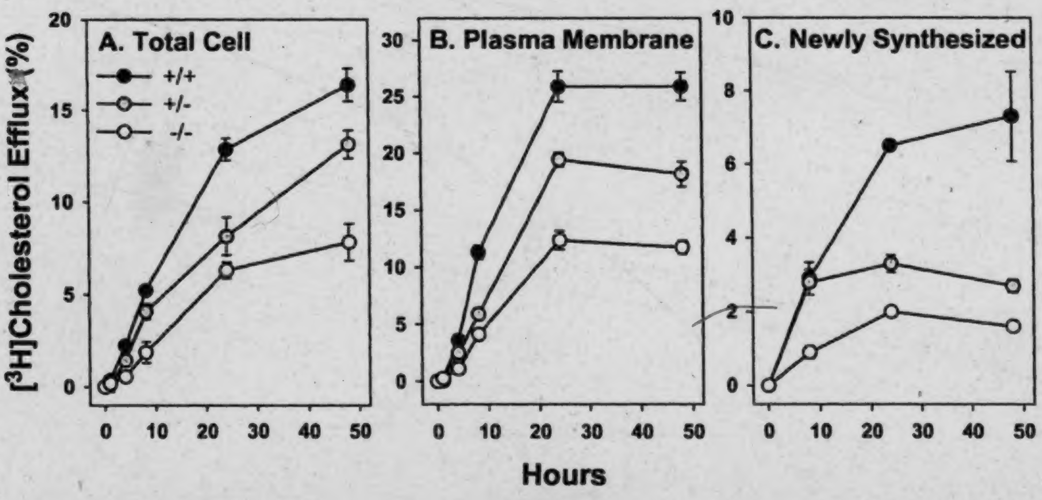


Figure 3-3. ApoA-I-mediated efflux of non-LDL-derived cholesterol from human NPC1-deficient fibroblasts. *A*, Cells labeled with [^{3}H]cholesterol during growth, loaded with unlabeled non-lipoprotein cholesterol for 24 h, and equilibrated for 24 h were incubated with apoA-I for 1–48 h to determine efflux of cellular cholesterol. Cell [^{3}H]cholesterol at time 0 h of apoA-I efflux was 407 ± 11, 477 ± 21, and 633 ± 35 x 10^3 dpm/mg cell protein for *NPC1*$^{+/+}$, *NPC1*$^{+/-}$, and *NPC1*$^{-/-}$ cells respectively. *B*, Cells were cholesterol-loaded for 24 h, equilibrated for 24 h, and then radiolabeled with [^{3}H]cholesterol for 2 h prior to incubation with apoA-1 for 1–48 h to determine efflux of plasma membrane cholesterol. Cellular [^{3}H]cholesterol at time 0 h of apoA-I efflux was 1049 ± 47, 2084 ± 165, and 1133 ± 64 x 10^3 dpm/mg cell protein for *NPC1*$^{+/+}$, NPC1$^{+/-}$, and *NPC1*$^{-/-}$ cells, respectively. *C*, Cells were radiolabeled with [^{14}C]mevalonic acid lactone during the last 40% of growth to confluence, equilibrated for 24 h, and incubated with 10 µg/ml apoA-I for 8–48 h to assess efflux of newly synthesized cholesterol. Cell [^{14}C]cholesterol at time 0 h of apoA-I efflux was 10.8 ± 1.0, 10.4 ± 1.8, and 17.1 ± 1.7 x 10^3 dpm/mg cell protein for *NPC1*$^{+/+}$, NPC1$^{+/-}$, and *NPC1*$^{-/-}$ cells, respectively. In each panel the data are expressed as amount of labeled sterol effluxed to the medium (following subtraction of efflux to medium containing 1 mg/ml BSA alone) as a percentage of total labeled sterol in medium and cells. *A* and *B* represent averages ± S.D. of three experiments performed in quadruplicate. *C* shows the mean ± S.D. of quadruplicate determinations and is representative of two experiments with similar results. *Symbols* are as in Figure 3-2. For all panels, values at $\geq$ 4 h are lower for *NPC1*$^{-/-}$ cells than *NPC1*$^{+/+}$ cells; for *A* and *B*, values at $\geq$ 8 h are lower for *NPC1*$^{+/-}$ cells than *NPC1*$^{+/+}$ cells, and for *C*, at > 8 h. For all significant differences, $p \leq 0.05$. The figure was published in Choi H.Y. *et al.* (2003) *J Biol Chem.* 278: 32569-32577.

cells showed intermediate levels of PC efflux; SM efflux from *NPC1*$^{+/-}$ cells was similar to *NPC1*$^{+/+}$ cells at early time points (≤8 h) but fell to levels similar to those from *NPC1*$^{-/-}$ cells at later time points. Impaired efflux of choline-containing phospholipids by NPC1-deficient cells parallels the decreased ability of apoA-I to mobilize cholesterol from all of the cellular cholesterol pools examined (Figures 3-2 and 3-3).

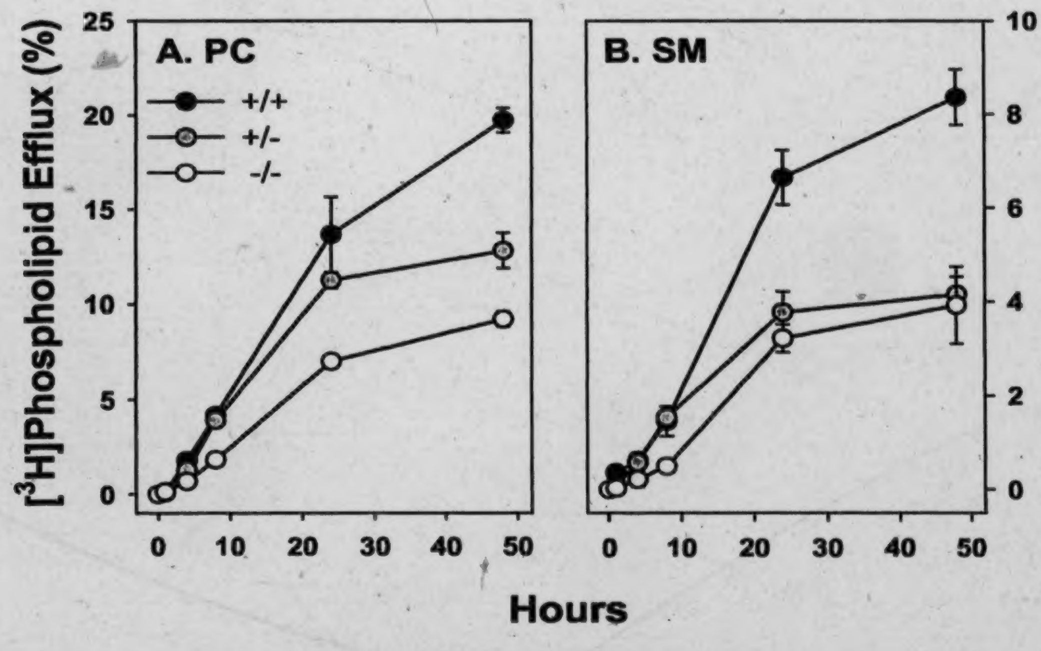


Figure 3-4. ApoA-I-mediated efflux of choline-containing phospholipids from human NPC1-deficient fibroblasts. Confluent cells were cholesterol-loaded for 24 h, radiolabeled with [^{3}H]choline chloride for 24 h, and then incubated with 10 µg/ml apoA-I for 1–48 h to determine efflux of phosphatidyl[^{3}H]choline (*PC*) (*A*) and [^{3}H]sphingomyelin (*SM*) (*B*). Total cell [^{3}H]choline at time 0 h of apoA-I efflux was 558 ± 8, 351 ± 39, and 573 ± 24 x 10^3 dpm/mg cell protein for *NPC1*$^{+/+}$, *NPC1*$^{+/-}$, and *NPC1*$^{-/-}$ cells, respectively. Values are the averages ± S.D. of three experiments performed in quadruplicate, expressed as the percentage of total cellular plus medium counts for PC or SM in the medium following subtraction of efflux to medium containing 1 mg/ml albumin alone. Symbols are as in Figure 3-2. For both panels, values at ≥ 4 h are lower for *NPC1*$^{-/-}$ cells than *NPC1*$^{+/+}$ cells, and values for *NPC1*$^{+/-}$ cells are lower than *NPC1*$^{+/+}$ cells at > 8 h ($p \leq 0.05$). The figure was published in Choi H.Y. *et al.* (2003) *J Biol Chem.* 278: 32569-32577.

3.3.5 ABCA1 expression is diminished in human NPC1$^{-/-}$ *fibroblasts*

Impaired efflux of phospholipids and various pools of cellular cholesterol to apoA-I from *NPC1*$^{-/-}$ fibroblasts suggests ABCA1 regulation and activity is also impaired in these cells. Levels of ABCA1 mRNA and protein were determined under non-cholesterol-loaded and cholesterol-loaded conditions. Semi-quantitative determination of *ABCA1* mRNA using reverse transcriptase-PCR was consistent with results obtained by Northern blotting (Figure 3-5A). ABCA1 mRNA and protein levels increased in *NPC1*$^{+/+}$ fibroblasts in response to non-lipoprotein cholesterol loading. *NPC1*$^{+/-}$ cells showed somewhat lower *ABCA1* mRNA levels by Northern blot and lower ABCA1 protein levels in response to cholesterol loading compared with *NPC1*$^{+/+}$ cells. In sharp contrast, *NPC1*$^{-/-}$ fibroblasts showed diminished basal and cholesterol-stimulated ABCA1 mRNA and protein levels when compared with *NPC1*$^{+/+}$ and *NPC*$^{+/-}$ cells. Although loading with cholesterol increased *ABCA1* expression in all cells, the amount of *ABCA1* mRNA and protein was much less in *NPC1*$^{-/-}$ cells, despite the fact that incorporation of both LDL-derived and non-lipoprotein cholesterol was higher in these cells (Figure 3-2 and 3-3 legends). A similar pattern of ABCA1 protein levels was seen in Western blots of LDL-loaded cells. *NPC1*$^{-/-}$ cells showed significantly lower ABCA1 protein levels than *NPC1*$^{+/+}$ cells both before and after loading with non-lipoprotein cholesterol (Figure 3-5B). Diminished *ABCA1* expression in *NPC*$^{-/-}$ cells is consistent with the decreased ability of these cells to mobilize phospholipids and cholesterol to apoA-I. The results strongly suggest that NPC1 protein function is required for the regulation and activity of ABCA1 and that the accumulation of cellular lipids in NPC1 disease fibroblasts results in part from diminished function of ABCA1.

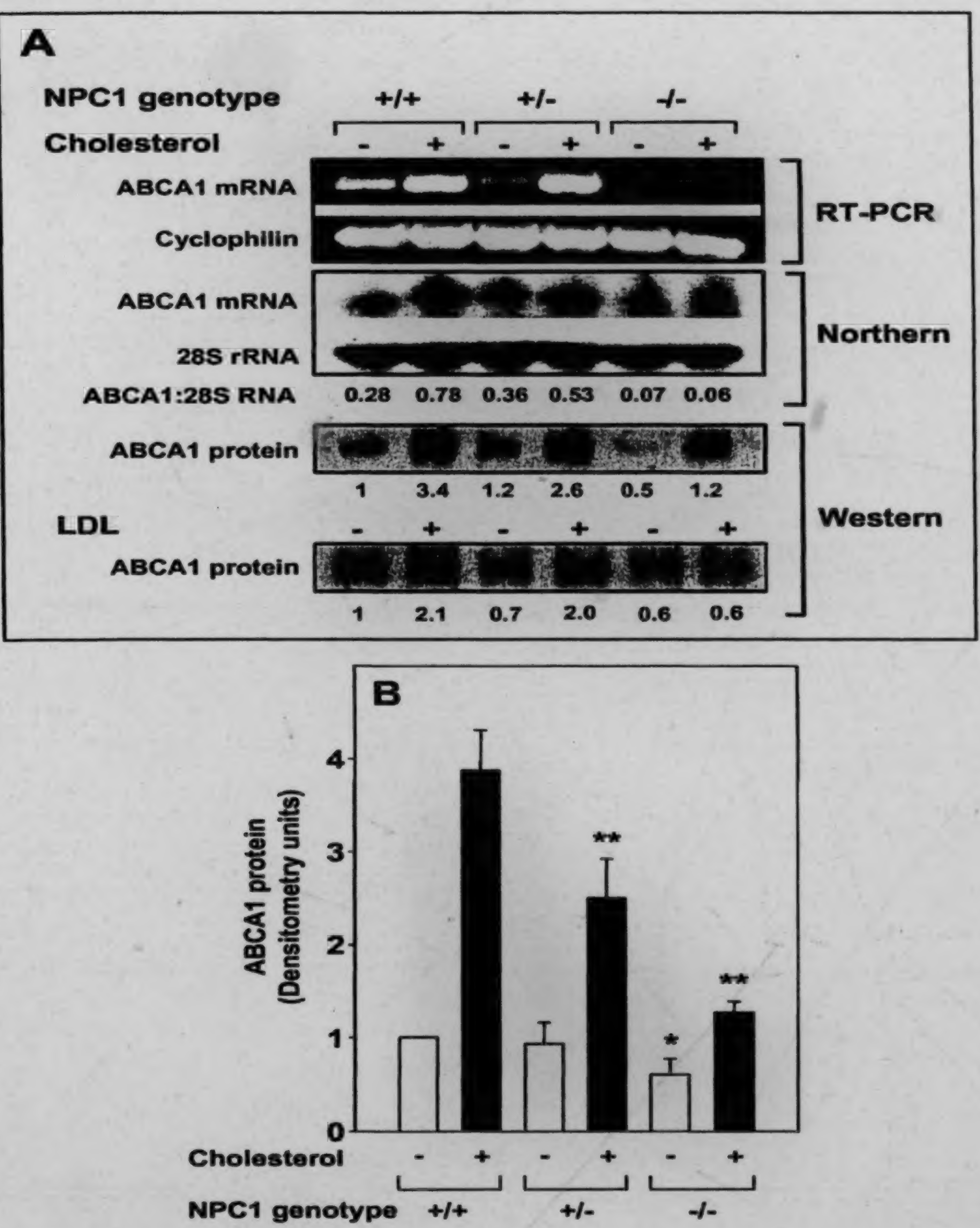


Figure 3-5. Expression of ABCA1 in human NPC1-deficient fibroblasts. *A*, cells were grown to confluence in DMEM containing 10% FBS, then incubated in the absence or presence of 30 μg/ml non-lipoprotein cholesterol for 24 h, and equilibrated in DMEM/BSA for 24 h prior to the determination of ABCA1 mRNA and protein levels. Alternatively, cells were grown the last 40% to confluence in lipoprotein-deficient serum and then incubated with 50 μg/ml LDL for 24 h.

Cyclophilin and 28 S rRNA were used as loading controls for reverse transcriptase-PCR and Northern blotting, respectively; the ratio of *ABCA1* mRNA to 28 S rRNA for the Northern blot is indicated. RNA determinations and Western blots are representative of two or more experiments each with similar results. ABCA1 protein was detected by Western blotting of 30 μg of cellular membrane protein with rabbit polyclonal anti-human ABCA1 antibody. Numeric values represent the intensities of ABCA1 protein bands relative to non-cholesterol loaded *NPC1*$^{+/+}$ cells. *B*, average ABCA1 protein levels as determined by Western blotting in cells incubated in the absence (–) or presence (+) of non-lipoprotein cholesterol, relative to non-cholesterol-loaded *NPC1*$^{+/+}$ cells. Results are averages ± S.D. for 3 experiments. *, $p < 0.05$ relative to non-cholesterol-loaded *NPC*$^{+/+}$ cells; **, $p < 0.001$ relative to cholesterol-loaded *NPC1*$^{+/+}$ cells. Average ABCA1 protein levels in cholesterol-loaded *NPC1*$^{-/-}$ cells are less than those in cholesterol-loaded *NPC1*$^{+/-}$ cells, $p < 0.01$. The figure was published in Choi H.Y. *et al.* (2003) *J Biol Chem.* 278: 32569-32577.

3.3.6 ABCA1 expression levels do not predict binding of ApoA-I to NPC-deficient fibroblasts

Lipid efflux to apoA-I has been shown to correlate directly with binding of apoA-I to cells [222] and with levels of *ABCA1* expression (reviewed in Ref. [210]). Cross-linking studies have suggested a direct protein-protein interaction between apoA-I and ABCA1 [114, 223, 224], and apoA-I binding appears to enhance ABCA1 activity by preventing its degradation by a calpain protease [132, 157]. To assess binding of apoA-I to NPC1-deficient cells, fibroblasts grown to confluence in 10% FBS were incubated in the presence or absence of non-lipoprotein cholesterol and then with ^{125}I-apoA-I. As expected from previous reports [222], binding of apoA-I was markedly higher to cholesterol-loaded (Figure 3-6A) than to non-cholesterol-loaded (Figure 3-6B) cells of all 3 *NPC1* genotypes. With both degrees of cholesterol loading, *NPC1*$^{+/-}$ cells showed the highest levels of apoA-I binding. Despite marked differences in ABCA1 protein levels in cholesterol-loaded and non-loaded conditions (Figure 3-5), *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ cells showed similar levels of apoA-I binding. The results with all three of these cell types suggest that other factors in addition to the amount of ABCA1 determine apoA-I binding to cells.

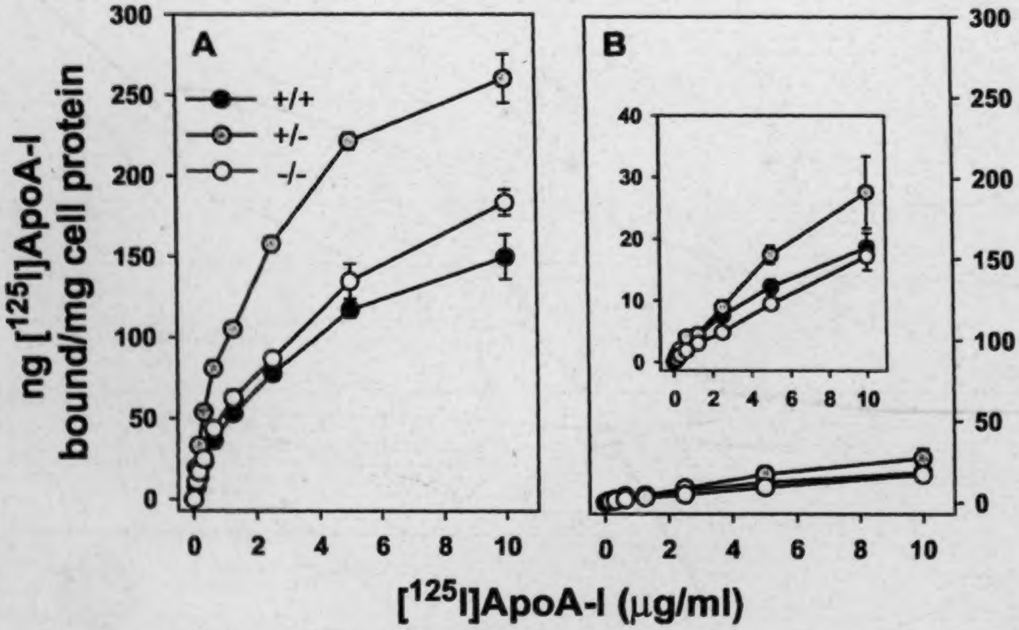


Figure 3-6. Binding of ^{125}I-apoA-I to human NPC1-deficient fibroblasts. Cells grown to confluence in DMEM, 10% FBS were incubated with (*A*) or without (*B*) non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h. Cells were then incubated with ^{125}I-apoA-I for 2 h at 0 °C. After extensive washing, cells were assessed for radioactivity. *B, inset* represents the data with the *y* axis expanded. Values represent the averages ± S.D. of three (*A*) and two (*B*) experiments performed in duplicate. Symbols are as in Figure 3-2. Values for *NPC1*$^{+/-}$ cells are greater than *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ cells at $\geq$ 0.625 µg/ml (*A*) or >2.5 µg/ml (*B*) ^{125}I-apoA-I ($p \leq 0.05$). The figure was published in Choi H.Y. *et al.* (2003) *J Biol Chem.* 278: 32569-32577.

3.3.7 HDL levels are low in $NPC1^{-/-}$ subjects

The results using human fibroblasts indicate impaired ABCA1-dependent HDL particle formation by NPC1-deficient cells in culture. Although the lipid profiles of NPC-deficient patients have been reported previously to be normal [204, 225], the only data in the literature were for total plasma cholesterol levels [226]. With the help of the Ara Parseghian Medical Research Foundation, we obtained the fasting lipid profiles of 21 $NPC1^{-/-}$ patients (Table 1). The majority of NPC patients are compound heterozygotes for NPC1 mutations [227]. Consistent with the finding of impaired ABCA1 expression in human $NPC1^{-/-}$ fibroblasts, we found that 9 of 10 male and 8 of 11 female subjects had HDL-cholesterol levels below the currently identified lower limit of normal for adults and children, 40 mg/dl or 1.03 mmol/liter (Figure 3-7) [228, 229]. The very high prevalence of low HDL levels in $NPC1^{-/-}$ subjects is even more striking given that children normally have higher HDL levels than adults. HDL-cholesterol levels fall by an average of 14% in males and 5% in females following puberty [230]. The Bogalusa Heart Study of 4074 children reported average HDL-cholesterol levels in pre-pubertal Caucasian children ages 5–9 of 1.73 ± 0.57 mmol/liter (mean ± S.D., n = 459) for boys and 1.69 ± 0.56 mmol/liter (n = 450) for girls [230]. In contrast, HDL levels for children aged 5–9 in our study were strikingly lower, 0.63 ± 0.21 for boys (mean ± S.D., $n = 5$) and 0.81 ± 0.24 ($n = 5$) for girls, $p < 0.005$ for both boys and girls compared with Bogalusa Heart Study children in this age group. Other than low HDL- cholesterol, no consistent abnormalities were found in the remaining plasma lipid parameters of $NPC1^{-/-}$ subjects (Table 1). Although 2 of the 21 subjects had mildly elevated plasma triglyceride levels, the low incidence of this finding suggests the absence of an association between hypertriglyceridemia and the low HDL-cholesterol of human NPC disease.

Fasting lipid profiles were also obtained for 31 parents of NPC subjects in this study. Of these, 4 of 15 male and 2 of 16 female heterozygotes had low HDL-cholesterol (0.93, 0.90, 0.90. 0.93, 0.88, and 0.77 mmol/liter, respectively). Again, no consistent abnormalities were found among the other lipid parameters in the NPC heterozygote profiles (data not shown).

Table 3-1. Plasma lipid profiles in NPC1$^{-/-}$ patients.

No.	Sex	Age	TC (mmol/L)	TG (mmol/L)	LDL (mmol/L)	HDL (mmol/L)
1	M	5	5.87	2.51	4.45	0.28 *
2	M	7	4.09	2.28	2.38	0.67 *
3	M	7	3.39	0.94	2.12	0.83 *
4	M	9	2.79	1.49	1.27	0.67 *
5	M	9	3.08	1.37	1.76	0.70 *
6	M	11	4.09	0.98	2.38	1.27
7	M	17	3.78	1.26	2.33	0.88 *
8	M	33	4.22	1.74	2.48	0.93 *
9	M	40	3.44	0.98	2.35	0.83 *
10	M	42	3.05	0.71	1.99	0.78 *
11	F	3	3.65	1.43	2.46	0.54 *
12	F	5	3.59	1.87	2.15	0.59 *
13	F	7	5.5	1.1	3.9	1.09
14	F	8	2.87	1.2	1.47	0.85 *
15	F	8	3.72	1.13	2.22	0.98 *
16	F	10	3.49	1.31	2.28	0.62 *
17	F	11	3.39	3.42	1.22	0.59 *
18	F	17	5.59	2.28	4.45	0.91 *
19	F	18	3.49	0.93	1.66	1.42
20	F	20	4.78	2.19	2.97	0.80 *
21	F	32	3.39	2.21	1.19	1.19

* HDL level lower than normal range. TC, total cholesterol; TG, triglycerides. The Table was published in Choi H.Y. *et al.* (2003) *J Biol Chem.* 278: 32569-32577.

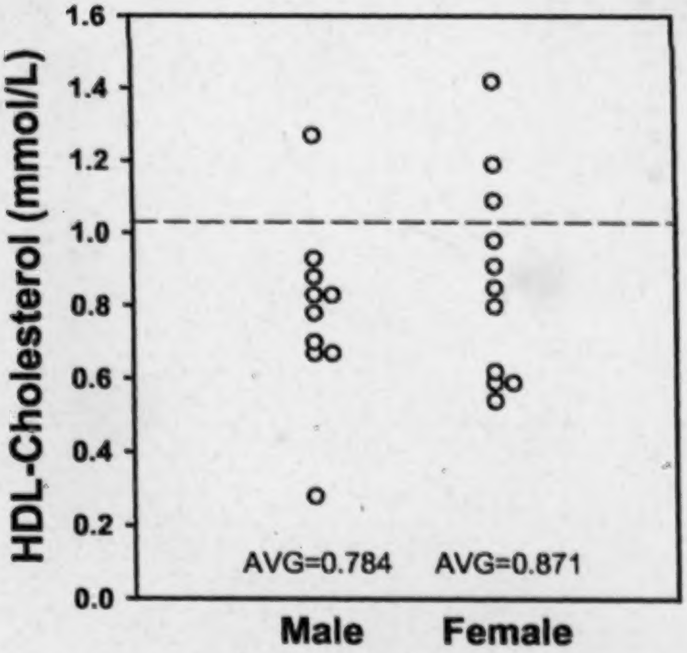


Figure 3-7. Plasma HDL levels in NPC1-deficient subjects. HDL-cholesterol levels (mmol/liter) obtained for male and female subjects are taken from Table I. The *dashed line* represents the lower limit of normal of HDL-cholesterol for children and adults (1.03 mmol/liter) [228, 229]. The figure was published in Choi H.Y. *et al.* (2003) *J Biol Chem.* 278: 32569-32577.

3.4 Further work done in our laboratory

The decreased expression and activity of ABCA1 despite accumulation of excess unesterified cholesterol in human *NPC1*$^{-/-}$ fibroblasts indicates that *NPC1*$^{-/-}$ cells may be impaired in the production or transport of oxysterols, which are normally produced in cholesterol-loaded cells [145, 231], and induce expression of genes involved in cholesterol efflux such as ABCA1 via activation of LXR (reviewed in [232]). In support of this concept, Frolov et al. [231] recently reported that the production of 25- and 27-hydroxycholesterol was impaired in human *NPC1*$^{-/-}$ fibroblasts, leading to the failure of LXR-mediated cellular responses. Frolov et al. [231] also showed that exogenously added 25- or 27-hydroxycholesterol to *NPC1*$^{-/-}$ cells significantly reduced cholesterol accumulation measured by cholesterol mass and filipin staining. These results, combined with earlier findings by Liscum *et al.* [177] and Lange *et al.* [233], suggest that correction of oxysterol-regulated gene expression may normalize the cholesterol trafficking defect caused by NPC1 deficiency.

In order to examine the effect of LXR activation more specifically, our laboratory tested whether treatment of *NPC1*$^{-/-}$ cells with a non-oxysterol synthetic LXR agonist, TO-901317, would correct the regulation and functions of ABCA1 and therefore HDL particle formation. I contributed to these studies by performing mRNA analysis and cholesterol mass assays, preparing experimental materials such as radiolabeled LDL and apoA-1, and advising on experimental techniques.

3.4.1 Diminished ABCA1 expression in NPC1$^{-/-}$ *human fibroblasts is corrected by LXR agonists*

To confirm whether exogenous LXR ligands can correct ABCA1 expression in *NPC1*$^{-/-}$ fibroblasts, cells were incubated with TO-901317 or 25- or 27-hydroxycholesterol. Again, basal and cholesterol-stimulated ABCA1 protein levels were low in *NPC1*$^{-/-}$ cells compared to *NPC1*$^{+/+}$ cells. However, treatment with LXR ligands induced a similar increase of ABCA1 protein in *NPC1*$^{-/-}$ and *NPC1*$^{+/+}$ cells relative to non-treated *NPC1*$^{+/+}$ cells, and TO-901317-stimulated

NPC1$^{-/-}$ cells showed a slightly higher ABCA1 level than cholesterol-stimulated *NPC1*$^{+/+}$ cells (Figure 3-8).

LXR ligand-stimulated mRNA levels of ABCA1 in *NPC1*$^{-/-}$ cells had been examined by reverse transcription PCR long before these studies were planned. At that time, 22(R)- and 27-hydroxycholesterol were tested as LXR ligands and a retinoid X receptor (RXR) ligand, 9-*cis* retinoic acid, was also tested since LXR functions with its obligate heterodimer partner RXR to activate target genes. *NPC1*$^{-/-}$ cells showed increased ABCA1 mRNA in response to exogenously added LXR or RXR ligands (Figure 3-9). These results indicate that the *ABCA1* gene in *NPC1*$^{-/-}$ cells is normally regulated in the presence of LXR/RXR ligands.

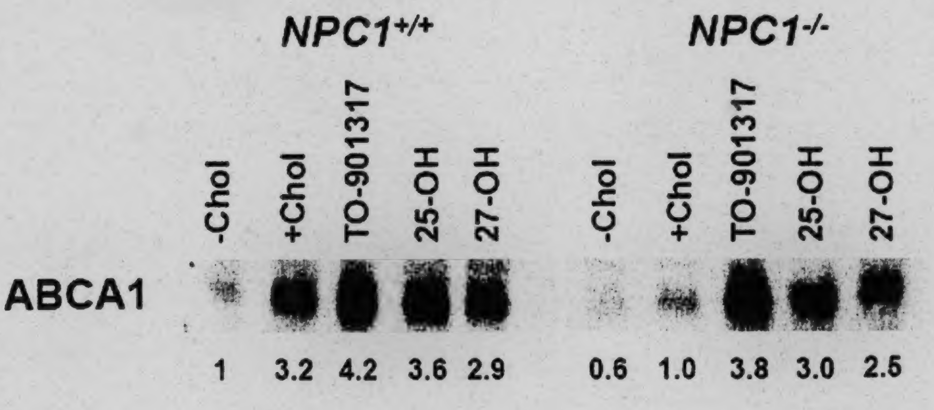


Figure 3-8. Correction of ABCA1 expression in NPC1$^{-/-}$ human fibroblasts treated with LXR agonists

. *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ fibroblasts were grown to confluence in DMEM/10% FBS and then incubated in the absence (-Chol) or presence of non-lipoprotein cholesterol 30 µg/ml (+Chol) in DMEM/BSA for 24 h. Non-cholesterol loaded cells were then equilibrated in DMEM/BSA plus 5 µM TO-901317 (TO-901317), 2.5 µM 25-hydroxycholesterol (25-OH), or 0.25 µM 27-hydroxycholesterol (27-OH) for 24 h prior to determination of ABCA1 protein by Western blot. ABCA1 protein was detected using 30 µg cellular membrane protein. Numeric values represent the intensities of ABCA1 protein bands relative to non-cholesterol loaded *NPC1*$^{+/+}$ cells. Results are representative of two separate experiments with similar results.

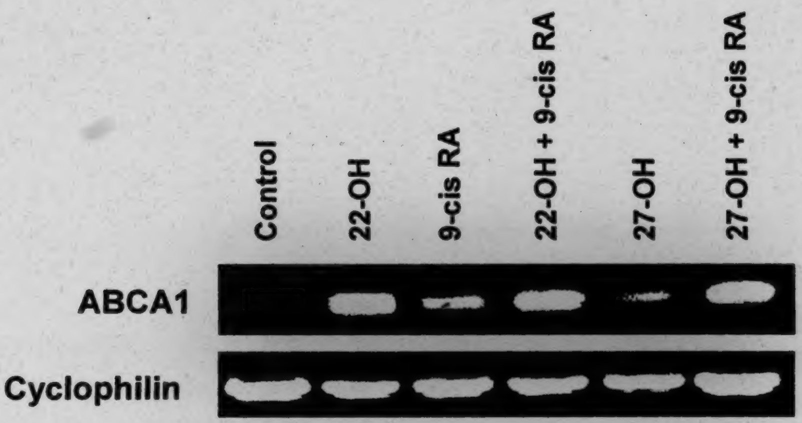


Figure 3-9. Normal up-regulation of ABCA1 mRNA in NPC1$^{-/-}$ human fibroblasts treated with LXR/RXR agonists. *NPC1*$^{-/-}$ fibroblasts were grown to confluence in DMEM/10% FBS and then incubated in DMEM/BSA alone (control) or with 10 µM 22(R)-hydroxycholesterol (22-OH), 10 µM 9-*cis* retinoic acid (9-*cis* RA), 10 µM 22-OH + 10 µM 9-*cis* RA, 10 µM 27-hydroxycholesterol (27-OH), or 10 µM 27-OH + 10 µM 9-*cis* RA for 24 h prior to determination of ABCA1 mRNA by reverse transcription PCR. Results are representative of two separate experiments with similar results.

3.4.2 ApoA-I mediated efflux of LDL-derived cholesterol and phosphatidylcholine is increased in NPC1$^{-/-}$ *fibroblasts treated with TO-901317*

To determine whether the activation of LXR corrects impaired lipid efflux from *NPC1*$^{-/-}$ cells to apoA-I, efflux of [^{3}H]-labeled cholesterol derived from LDL and [^{3}H]PC derived from [^{3}H]choline to apoA-I from cells were examined in the presence of TO-901317. These experiments were done by Diana Lee and Teddy Chan in our laboratory. TO-901317-stimulated *NPC1*$^{-/-}$ cells significantly increased efflux of the radiolabeled LDL-derived cholesterol and PC to apoA-I compared to non-stimulated *NPC1*$^{-/-}$ cells, reaching 83% of cholesterol and 103% of PC efflux from non-stimulated *NPC1*$^{+/+}$ cells (Figure 3-10). These results suggest that the activation of LXR may be sufficient to promote lipid efflux to apoA-I even in the presence of *NPC1* mutations. Efflux of cholesterol and phospholipids to lipid-free apoA-I is mediated mainly by ABCA1, as mentioned in Section 1.1.4.3. It is therefore thought that TO-901317-stimulated increased expression of ABCA1 is responsible for this effect.

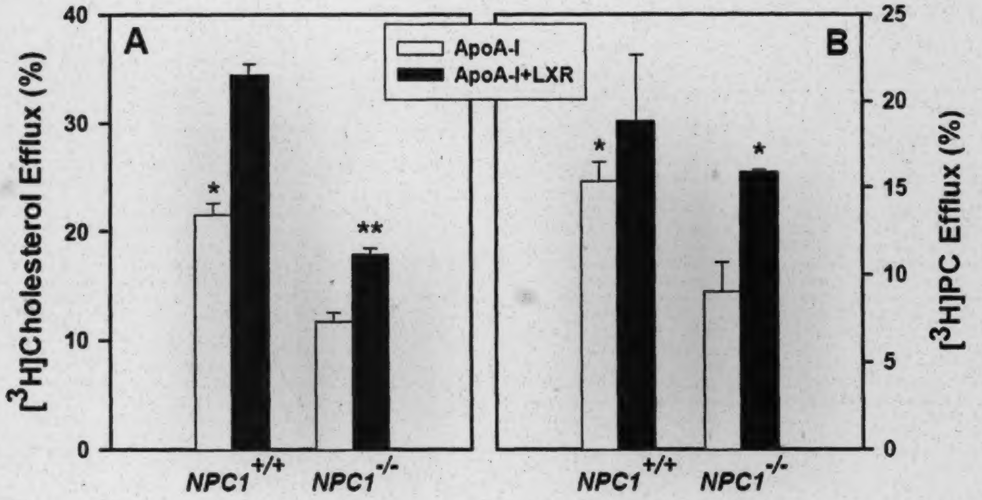


Figure 3-10. LXR agonist TO-901317 increases apoA-I mediated efflux of LDL-derived cholesterol and phosphatidylcholine from NPC1$^{-/-}$ human fibroblasts. *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ fibroblasts were labeled with [^{3}H]cholesteryl linoleate-labeled LDL or [^{3}H]choline and incubated with DMEM/BSA for 24 h followed by 10 µg/ml apoA-I for 24 h in the absence (white bars) or presence (black bars) of 5 µM TO-901317 (LXR). Results are expressed as percent of total cell plus medium [^{3}H]cholesterol (**A**) or [^{3}H]phosphatidylcholine (PC, **B**) in the medium. Total cell plus medium [^{3}H]cholesterol was 2566 ± 832, 2500 ± 79, 4793 ± 989, and 5295 ± 409 X 10^4 dpm/mg protein and of [^{3}H]choline was 4389 ± 566, 5993 ± 306, 7229 ± 892, and 8619 ± 883 X 10^4 dpm/mg protein for apoA-I-treated *NPC1*$^{+/+}$ cells, apoA-I-TO-901317-treated *NPC1*$^{+/+}$ cells, apoA-I-treated *NPC1*$^{-/-}$ cells, and apoA-I-TO-901317-treated *NPC1*$^{-/-}$ cells, respectively. Panel A: *, efflux greater than apoA-I-treated *NPC1*$^{-/-}$ cells, $p < 0.0001$, and greater than apoA-I-TO-901317-treated *NPC1*$^{-/-}$ cells, $p < 0.001$; **, efflux greater than apoA-I-treated *NPC1*$^{-/-}$ cells, $p < 0.001$. Panel B: *, efflux greater than apoA-I-treated *NPC1*$^{-/-}$ cells, $p < 0.01$ for both. Values are the means ± S.D. of quadruplicate determinations and are representative of three experiments with similar results.

3.4.3 ApoA-I mediated efflux of LDL-derived cholesterol mass is increased in NPC1$^{-/-}$ fibroblasts treated with TO-901317

To determine whether efflux of LDL-derived radiolabeled cholesterol to apoA-I is correlated with cholesterol mass efflux, we next determined the efflux of LDL-derived cholesterol mass to apoA-I in the absence or presence (following correction of ABCA1 expression) of TO-901317. *NPC1*$^{-/-}$ fibroblasts showed significantly higher cholesterol mass efflux than *NPC1*$^{+/+}$ fibroblasts in the control medium of DMEM containing 1 mg/ml BSA (Figure 3-11A, white bars); however, cholesterol mass efflux in the presence of 10 µg/ml apoA-I for 24 h was similar to *NPC1*$^{+/+}$ fibroblasts (Figure 3-11A, hatched bars). Therefore, apoA-I-dependent cholesterol mass efflux from *NPC1*$^{-/-}$ fibroblasts following subtraction of efflux to BSA-containing control medium was significantly lower than that from *NPC1*$^{+/+}$ fibroblasts (Figure 3-12). TO-901317-stimulated *NPC1*$^{-/-}$ fibroblasts markedly increased efflux of LDL-derived cholesterol mass to apoA-I (Figure 3-12). These results are similar to apoA-I mediated efflux of LDL-derived radiolabeled cholesterol (Figure 3-10).

Consistent with previous reports [174, 205], *NPC1*$^{-/-}$ fibroblasts loaded with LDL accumulated approximately 3.5 fold more UC when compared to *NPC1*$^{+/+}$ fibroblasts, and approximately 50% less CE mass in the control medium (Figure 3-11C and B, respectively, white bars). Incubation with 10 µg/ml apoA-I for 24 h significantly lowered cellular CE mass both in *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ fibroblasts (Figure 3-11B). TO-901317-stimulated *NPC1*$^{+/+}$ fibroblasts showed a further lowering of cellular CE mass (Figure 3-11B) and also significantly lowered cellular UC mass when compared to non-TO-901317 treated *NPC1*$^{+/+}$ fibroblasts in the presence of apoA-I (Figure 3-11C). TO-901317-stimulated *NPC1*$^{-/-}$ fibroblasts also lowered cellular UC mass when compared to non-TO-901317 treated *NPC1*$^{-/-}$ fibroblasts, however it did not reach to statistical significance due to a large error bar (Figure 3-11C). These results suggest that correction of ABCA1 expression in human *NPC1*$^{-/-}$ fibroblasts may decrease the accumulation of UC in NPC1 disease by promoting lipid efflux to apoA-I.

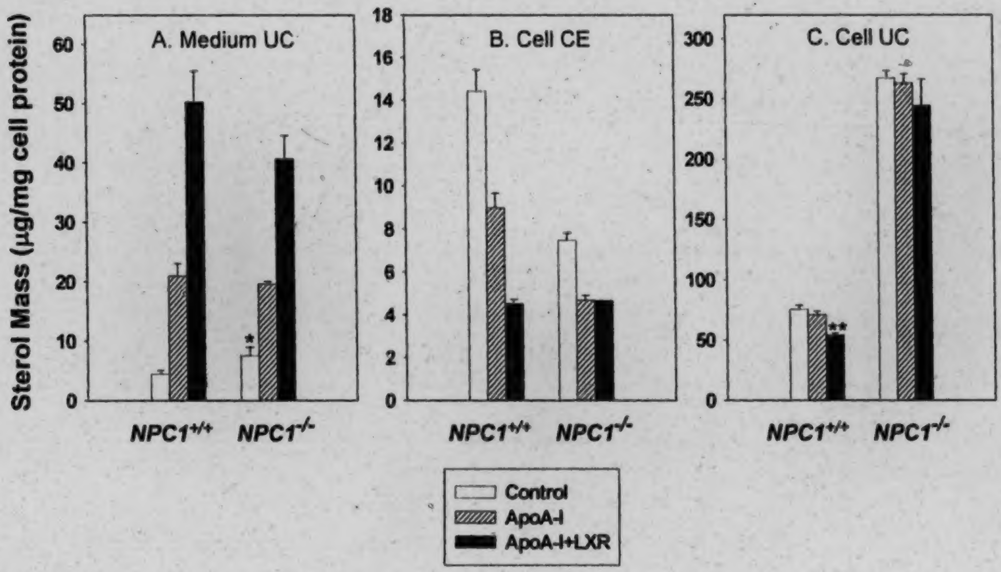


Figure 3-11. Efflux of LDL-derived cholesterol mass from human fibroblasts. *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ fibroblasts incubated in DMEM containing 10% lipoprotein-deficient serum during the last 40% of growth were loaded with 50 µg/ml LDL for 24 h and equilibrated for 24 h with or without 5 µM TO-901317. The cells were then incubated with medium containing 1 mg/ml BSA alone (control) or the same medium containing 10 µg/ml apoA-I in the absence or presence of 5 µM TO-901317 (LXR). At the end of the incubation, media and cells were collected and total lipids were extracted. Unesterified cholesterol (UC) mass in the medium **(A)**, cellular cholesteryl ester (CE) mass **(B)**, and cellular UC mass **(C)** were determined by gas chromatography. Panel A: *, mass greater than control *NPC1*$^{+/+}$ cells, $p < 0.05$. Panel C: **, mass less than control and apoA-I-treated *NPC1*$^{+/+}$ cells, $p < 0.01$. Results represent averages ± S.D. of a single experiment performed in triplicate.

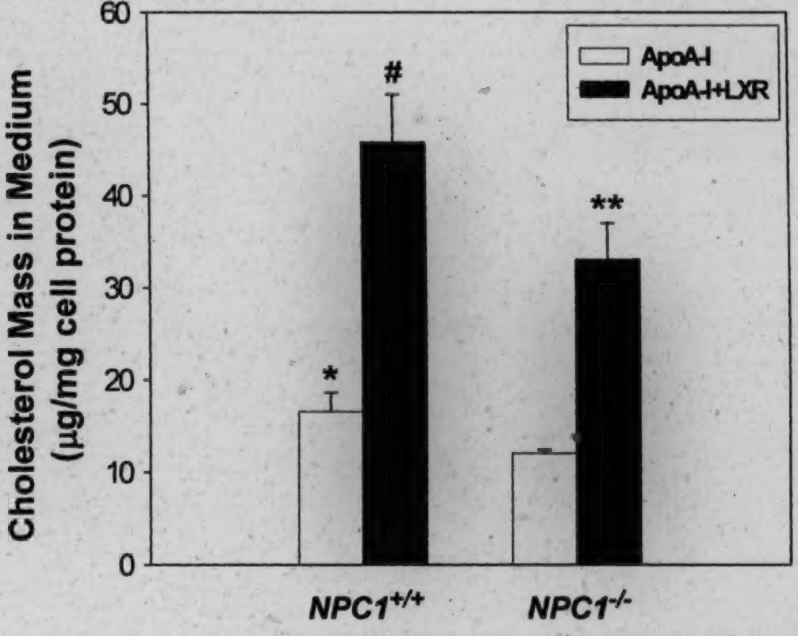


Figure 3-12. ApoA-I-dependent efflux of LDL-derived cholesterol mass from human fibroblasts. ApoA-I-dependent efflux of LDL-derived cholesterol mass was determined using the data from Figure 3-11 and subtracting cholesterol mass in the medium containing BSA alone of control cells from cholesterol mass in the medium containing BSA plus apoA-I or apoA-I plus TO-901317-treated cells. *, mass greater than apoA-I-treated *NPC1*$^{-/-}$ cells, $p < 0.05$; #, mass greater than apoA-I plus TO-901317-treated *NPC1*$^{-/-}$ cells, $p < 0.05$; **, mass greater than apoA-I-treated *NPC1*$^{-/-}$ cells, $p < 0.001$.

3.4.4 Correction of apoA-I-induced HDL particle formation by NPC1$^{-/-}$ fibroblasts treated with TO-901317

ApoA-I-containing HDL is present in various forms in human plasma. HDL subspecies differ in apolipoprotein and lipid composition, size, and charge. When separated by two-dimensional non-denaturing gel electrophoresis on the basis of charge and size differences, HDL subspecies are classified as small pre β mobility (pre β-1 and pre β-2) and large α mobility (α–1, α–2, and α–3) particles (Figure 3-13) [234].

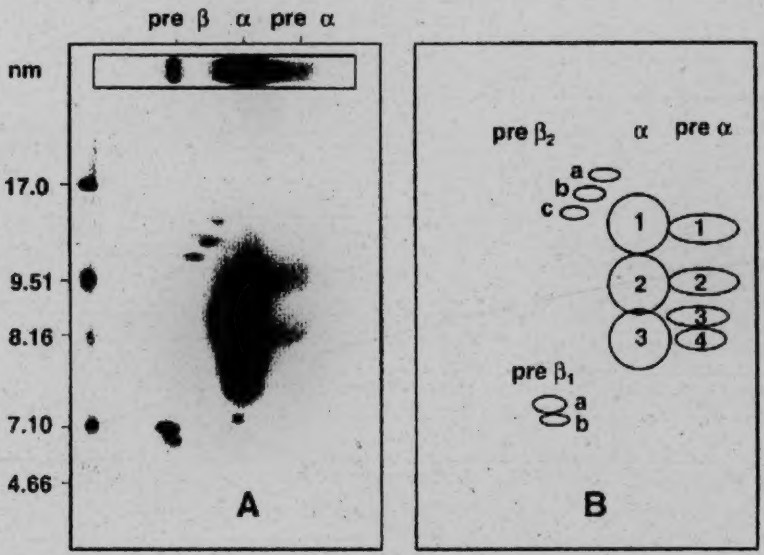


Figure 3-13. ApoA-I-containing HDL subpopulations determined by two-dimensional non-denaturing gel electrophoresis, immunoblot and image analysis. Panel A shows the apoA-I-containing HDL subpopulations of a normolipidemic male subject (HDL=47.5 mg/dl). The rectangular insert represents the first-dimensional separation on agarose gel. A duplicate of the agarose strip was applied on the top of a 3–35% concave gradient gel and electrophoresed in the second dimension. Molecular weight standards are on the left side of panel A. Panel B is a schematic representation of individual subpopulations in human plasma. The figure was taken from B.F. Asztalos and E.J. Schaefer [234] and is presented here with permission.

To determine the effect of TO-901317 treatment on HDL particle formation by cultured $NPC1^{-/-}$ cells, cell-conditioned medium was analyzed for HDL species following incubation with apoA-I in the absence or presence of TO-901317. These experiments were done by Emmanuel Boadu in our laboratory. Two-dimensional gel electrophoresis showed $NPC1^{-/-}$ cells generate fewer large α mobility particles than $NPC1^{+/+}$ cells in the absence of TO-901317 (Figure 3-14A). Treatment with TO-901317 corrected the generation of large α mobility particles by $NPC1^{-/-}$ cells to a pattern similar to that seen in non-TO-901317-treated $NPC1^{+/+}$ cells (Figure 3-14B).

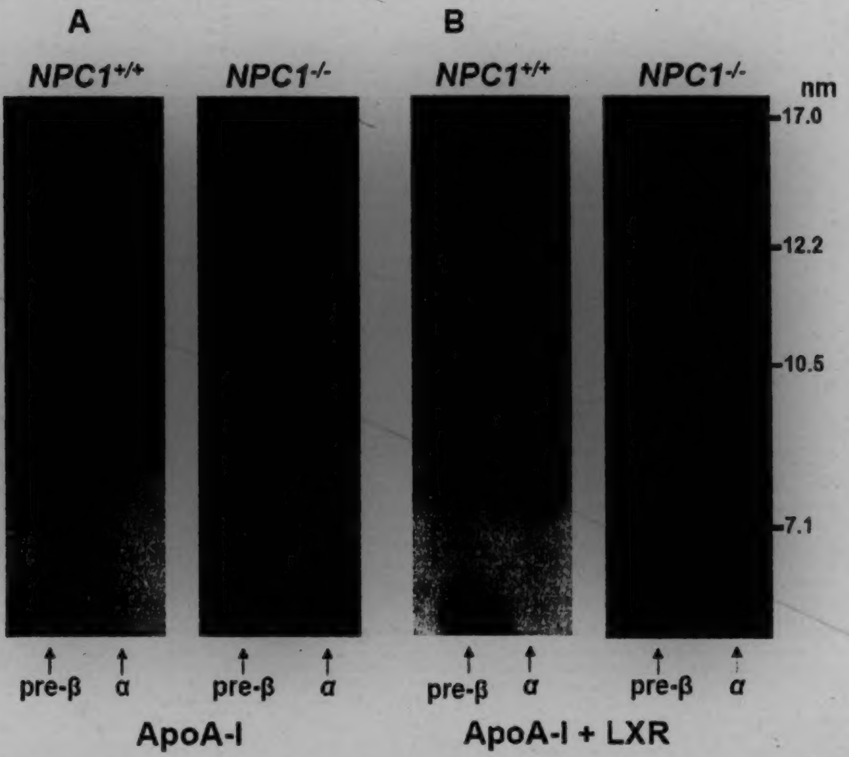


Figure 3-14. Correction of HDL particle formation by TO-901317-treated human $NPC1^{-/-}$ fibroblasts. $NPC1^{+/+}$ and $NPC1^{-/-}$ fibroblasts incubated in 10% lipoprotein-deficient serum during the last 40% growth were incubated for 24 h with 50 µg/ml LDL. The cells were then equilibrated for 24 h and incubated for 24 h with 10 µg/ml apoA-I in the absence (**A**) or presence (**B**) of 5 µM TO-901317 (LXR). Media from the cells were collected and analyzed for HDL particle species by two-dimensional non-denaturing gel electrophoresis and Western blotting with anti-human apoA-I antibody. Results are representative of two separate experiments with similar results.

These results suggest that the *NPC1* mutation-induced impairment of HDL particle formation can be corrected by exogenous TO-901317, which provides a potential therapeutic mechanism for correction of lipid accumulation and trafficking in NPC disease.

3.5 Discussion

Niemann-Pick type C disease is characterized by the accumulation of cholesterol in late endosomes/lysosomes and an inability to regulate normally three central mechanisms of cholesterol homeostasis: delivery of unesterified cholesterol to the endoplasmic reticulum for esterification by acyl-CoA:cholesterol acyltransferase, regulation of cholesterol synthesis by 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and regulation of LDL receptor activity [174, 176, 177, 204]. In the current studies, we demonstrate that regulation of another pivotal mediator of cholesterol homeostasis, *ABCA1*, is also impaired in human NPC1-deficient fibroblasts. ApoA-I showed a diminished ability to mobilize radiolabeled cholesterol in *NPC1*$^{-/-}$ cells from LDL-derived and non-lipoprotein-derived cholesterol pools, and to mobilize radiolabeled cellular phosphatidylcholine and sphingomyelin. ABCA1 mRNA and protein levels in *NPC1*$^{-/-}$ cells were diminished at basal levels of cell cholesterol and following loading of cells with either non-lipoprotein- or LDL-derived cholesterol, when compared with *NPC*$^{+/+}$ and *NPC*$^{+/-}$ cells. Consistent with impaired regulation of *ABCA1* at the cellular level, we found a strikingly high incidence of hypoalphalipoproteinemia (90% of males and 73% of females) in the lipid profiles of 21 *NPC1*$^{-/-}$ subjects.

Impaired activity of ABCA1 in NPC-deficient cells is strongly suggested by the diminished basal and cholesterol-stimulated levels of ABCA1 mRNA and protein, and decreased levels of phospholipid and cholesterol efflux to apoA-I from these cells. The pattern of accumulation of cell cholesterol in NPC disease and localization of the NPC1 protein has led to the conclusion that the major site of action of NPC1 is in late endosomes/lysosomes [164, 206]. ABCA1 mobilizes cellular lipids to apoA-I at the plasma membrane [210, 235] and may also facilitate the delivery of intracellular lipids to internalized or cell surface apolipoproteins from late endosomes/lysosomes [109, 110, 113, 236]. As such, mutations in *NPC1* might adversely affect the function of ABCA1 in facilitating the removal of late endosomal/lysosomal cholesterol. We found the greatest degree of inhibition of apoA-I-mediated cholesterol mobilization from *NPC1*$^{-/-}$ cells from

LDL-derived cholesterol (Figure 3-2), which accumulates mainly in late endosomes/lysosomes in these cells [171]. We also found a >50% decrease in cholesterol mobilization to apoA-I from non-lipoprotein-derived cholesterol pools, including newly synthesized cholesterol, in *NPC1*$^{-/-}$ cells (Figure 3-3). Although the initial delivery of newly synthesized cholesterol to the plasma membrane is normal in NPC cells [171, 207, 237], subsequent trafficking of this cholesterol back to intracellular compartments and therefore mobilization to apoA-I may be impaired in the presence of *NPC1* mutations. Of the several cholesterol labeling methods utilized, efflux to apoA-I of cholesterol from cells pulse-labeled with [^{3}H]cholesterol may represent the pathway least dependent on NPC1, as NPC1 is not currently known to function directly in the plasma membrane. ABCA1, on the other hand, is thought to function, at least in part, at the cell surface to deliver lipids to apoA-I. Impaired efflux of cholesterol from *NPC1*$^{-/-}$ cells labeled using this method therefore provides further evidence for decreased ABCA1 activity in *NPC1*$^{-/-}$ cells, and for ABCA1 mobilizing cholesterol from plasma membrane as well as late endosomal/lysosomal pools.

Intermediate levels of esterification of LDL-derived cholesterol have been reported previously [205] in heterozygous *NPC1* cells during the first 6 h of incubation with LDL, with normal levels of esterification in these cells incubated over 24 h with LDL. We found similar overall levels of esterification and efflux to apoA-I of LDL-derived [^{3}H]cholesterol in *NPC1*$^{+/-}$ and *NPC1*$^{+/+}$ cells following a 24-h incubation with labeled LDL (Figure 3-2). Efflux of total cellular, plasma membrane, and newly synthesized [^{3}H]cholesterol from *NPC1*$^{+/-}$ cells were intermediate between *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ cells (Figure 3-3), as was efflux of phosphatidylcholine (Figure 3-4). Northern blot analysis indicated a moderate decrease in cholesterol-induced levels of *ABCA1* mRNA in *NPC1*$^{+/-}$ relative to *NPC1*$^{+/+}$ cells, whereas ABCA1 protein levels in response to cholesterol and LDL loading were similar between *NPC1*$^{+/+}$ and *NPC1*$^{+/-}$ cells (Figure 3-5). We found low HDL in 6 out of 31 *NPC1* heterozygote lipid profiles studied, which is likely more than expected in the general population. The ability of *NPC1*$^{+/-}$ cells to mediate ABCA1-dependent lipid efflux might vary based on heterogeneity of

NPC1 mutations, but intermediate levels of lipid efflux to apoA-I from *NPC1*$^{+/-}$ cells shown in this study may be useful in predicting low HDL formation or plasma levels of HDL in NPC heterozygotes.

The markedly decreased *ABCA1* expression and decreased ABCA1-dependent lipid efflux to apoA-I in the classic *NPC1*$^{-/-}$ phenotype cells studied, however, and the low HDL-cholesterol levels in the vast majority (81%) of *NPC1*$^{-/-}$ patients studied (Table 3-1 and Figure 3-7) do indicate the strong likelihood of impaired *ABCA1* regulation in NPC disease subjects with the classic biochemical phenotype. This very high incidence of hypoalphalipoproteinemia suggests these results cannot be explained by chance. Although the incidence of heterozygous ABCA1 mutations in the general population is unknown, they are unlikely to represent a frequent cause of low HDL-cholesterol [238]. ABCA1-mediated lipidation of apoA-I is now widely accepted to be the rate-limiting step in HDL particle formation and a key predictor of circulating HDL levels [210, 239]. Therefore, impaired regulation of ABCA1 activity, as indicated by the lipid efflux results and *ABCA1* expression levels in human *NPC1*$^{-/-}$ cells, is the most likely explanation for such a high incidence of low HDL-cholesterol values in NPC disease. The absence of low HDL in all the NPC patient lipid profiles obtained is likely an additional demonstration of the known heterogeneity of biochemical and clinical presentations in this disorder [240, 241], which would include variable regulation of *ABCA1* expression. The very high incidence of low HDL in NPC disease patients, however, suggests this could be used as an additional diagnostic criterion to help rule in or out Niemann Pick C disease in children, which is frequently a difficult diagnosis.

The ability of an exogenous LXR agonist, TO-901317, to increase *ABCA1* gene expression (Figure 3-8 and 3-9) and correct phospholipid and cholesterol efflux to apoA-I (Figure 3-10) for HDL particle formation (Figure 3-14) in the presence of *NPC1* mutations provides additional evidence that impaired regulation of ABCA1 is the most likely explanation for the low HDL-cholesterol levels shown in the majority of NPC patients. Correction of ABCA1 functions in *NPC1*$^{-/-}$ cells by TO-901317 is consistent with the known ability of exogenously added oxysterols to correct the defects in cholesterol esterification, cholesterol synthesis, LDL receptor activity, and lysosomal cholesterol accumulation in human $NPC1^{-/-}$ fibroblasts [177, 231, 233]. These results suggest that LXR agonists could be attractive molecules for correction of lipid accumulation and trafficking in NPC disease. When we consider that hepatic accumulation of UC is an important part of NPC disease [242] and hepatic ABCA1 is responsible for ~80% of HDL particle formation, at least in one mouse model [42], it seems to be crucial to increase hepatic ABCA1 expression in NPC patients. However, hepatic ABCA1 is reported to be much less responsive to LXR agonist stimulation compared with extrahepatic ABCA1 [243]. Therefore, the development of specific LXR agonists, which effectively stimulate hepatic ABCA1 expression, will likely determine the potential usefulness of LXR agonist treatment to correct lipid accumulation and low HDL-cholesterol in NPC disease.

Figure 3-12 showed that efflux of LDL-derived cholesterol mass from $NPC1^{-/-}$ fibroblasts was significantly lower than that from $NPC1^{+/+}$ fibroblasts, however, the extent of the decreased efflux of LDL-derived cholesterol mass was smaller than the decreased efflux of LDL-derived radiolabeled cholesterol (Figure 3-10A). This seems likely due to dilution of radiolabeled cholesterol in $NPC1^{-/-}$ fibroblasts; $NPC1^{-/-}$ fibroblasts accumulated approximately 3.5 fold more UC than $NPC1^{-/-}$ fibroblasts (Figure 3-11C), therefore, specific radioactivity (radiolabeled cholesterol mass divided by total cholesterol mass) will be much less in $NPC1^{-/-}$ cells than that in $NPC1^{+/+}$ cells, which may lead us to underestimate cholesterol efflux from $NPC1^{-/-}$ cells. $NPC1^{-/-}$ cells showed cholesterol mass efflux higher than $NPC1^{+/+}$ cells into the control medium of DMEM containing 1mg/ml BSA (Figure 3-11A). The reason for that result is not clear from this study, and it remains to be determined whether $NPC1^{-/-}$ cells have higher interaction capacity with BSA when compared with $NPC1^{+/+}$ cells. Even though apoA-I-treated $NPC1^{-/-}$ cells showed similar efflux of LDL-derived cholesterol to apoA-I-treated $NPC1^{+/+}$ cells before subtracting efflux to medium containing BSA alone (Figure 3-11A), apoA-I-treated $NPC1^{-/-}$ cells generated fewer and smaller HDL particles (Figure 3-14A). These results suggest that cholesterol released into BSA-containing medium from *NPC1*$^{-/-}$ cells does not contribute to HDL particle formation. The consistency between apoA-I-specific cholesterol mass efflux (Figure 3-12) and HDL particle formation (Figure 3-14) indicate that HDL particle formation is determined by apoA-I-dependent cholesterol efflux. Although Figure 3-11C showed that TO-901317-stimulated *NPC1*$^{-/-}$ cells lowered cellular UC mass when compared to non-TO-901317-treated *NPC1*$^{-/-}$ cells, it was not statistically significant because of a large error bar. Further studies with a longer apoA-I incubation period are required to determine whether correction of ABCA1 expression reduces the accumulation of UC in NPC disease significantly.

Although the normalization of ABCA1 expression and HDL particle formation by TO-901317-treatred *NPC1*$^{-/-}$ cells demonstrate the critical role of ABCA1 in HDL formation, we can not exclude possible roles of other lipid transporters in HDL particle formation. The traditional view of ABCA1 action has been that it mediates the initial delivery of phospholipids and/or cholesterol to lipid-free/poor apoA-I to form small nascent HDL particles, which subsequently enlarge following delivery of additional lipids from cells via non-ABCA1 dependent mechanisms, possibly facilitated by the actions of other lipid transporters including ABCG1 [60, 244], ABCG4 [60], and scavenger receptor class B type I (SR-BI) [71]. The presence of large HDL particles in the conditioned medium of TO-901317 and apoA-I-treated *NPC1*$^{-/-}$ cells (Figure 3-14) suggests that lipid transporters facilitating cholesterol efflux to pre-formed HDL could play a role in enlargement of small nascent HDL particles formed by ABCA1. SR-BI is, however, not considered to be LXR-responsive [245] and our laboratory previously found no SR-BI in human fibroblasts [246]. *ABCG1* and *ABCG4* are LXR responsive genes [60, 244], and their expression in human fibroblasts has not been reported. Therefore, our laboratory determined levels of ABCG1 and ABCG4 mRNA in human *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ fibroblasts with positive controls for ABCG1 expression (mouse liver and brain [247]) and ABCG4 expression (mouse brain [248]). Reverse transcription PCR analyses showed that *NPC1*$^{-/-}$ fibroblasts express low basal levels of ABCG1 compared to

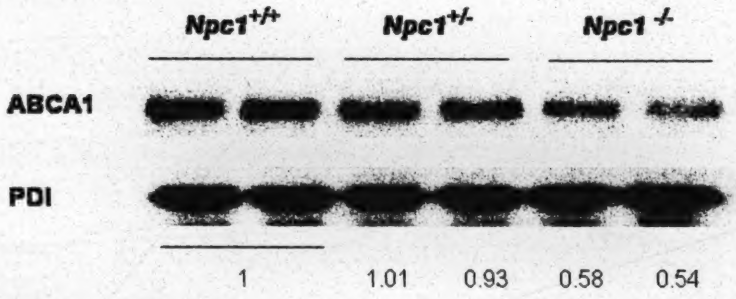
NPC1$^{+/+}$ fibroblasts with an increase upon addition of TO-901317 to levels similar to TO-901317-treated *NPC1*$^{+/+}$ fibroblasts, and that ABCG4 is not detected in human fibroblasts. These results suggest that the regulation of ABCG1 is also impaired in human *NPC1*$^{-/-}$ fibroblasts, and TO-901317-stimulated ABCG1 expression may contribute to the correction of HDL particle formation.

ABCA1 is thought to mobilize cellular phospholipids and cholesterol to apoA-I mainly from the plasma membrane [210], but may also facilitate the mobilization of late endosomal/lysosomal pools of lipids to apoA-I internalized along with ABCA1 and then resecreted at the plasma membrane as nascent HDL [109, 110, 113]. If enhanced ABCA1 activity effectively mobilized cellular cholesterol and phospholipids even in the presence of dysfunctional NPC protein, it would be important to determine whether this was a consequence of mobilization of plasma membrane lipids and secondary mobilization of lipid stores in the late endosome/lysosome compartment, or from direct mobilization of lipids by ABCA1 in this intracellular compartment. Lange *et al.* [233] speculated previously that the effect of exogenous oxysterols might be to cause a shift of lysosomal cholesterol to the plasma membrane following decreased cholesterol synthesis in oxysterol-treated *NPC1*$^{-/-}$ cells. The same could be true following ABCA1-mediated mobilization of plasma membrane cholesterol in TO-901317-treated cells, or a combination of this plus mobilization of intracellular lipid pools by ABCA1. Further studies are required to address the effects of specific upregulation of ABCA1 on cholesterol mobilization in NPC deficient cells.

Interestingly, *NPC1*$^{+/-}$ fibroblasts showed the highest levels of ^{125}I-apoA-I binding in both non-cholesterol-loaded and cholesterol-loaded cells (Figure 3-6). In addition, despite marked differences in *ABCA1* expression, levels of apoA-I binding to *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ cells were similar under both conditions. The reasons for this are unclear; however, they strongly suggest that factors other than ABCA1 are important in facilitating the apoA-I-cell interaction. These results suggest NPC cells may be an excellent model to study other key determinants of apoA-I binding.

Of note, Chen *et al.* [236] reported that macrophages from a murine model of NPC disease were impaired in cholesterol efflux but not in phospholipid efflux to apoA-I. This is in contrast to our results showing impaired efflux of both cholesterol and phospholipids from human *NPC1*$^{-/-}$ fibroblasts. Chen *et al.* [236] reported similar levels of induction of AbcA1 mRNA and protein for *Npc1*$^{-/-}$ and wild type mouse macrophages in response to treatment with LXR/RXR agonists, which is consistent with our findings in human fibroblasts. However, the Abca1 data were not shown and basal levels of *Abca1* expression were not indicated [236]. Low HDL-cholesterol levels found in the majority of NPC patients in our study are also in contrast to normal plasma HDL-cholesterol levels in BALB/c *Npc1*$^{-/-}$ mice [249, 250]. When we measured ABCA1 protein levels in 5 week old BALB/c *Npc1*$^{-/-}$ mice, liver ABCA1 protein levels were decreased to ~55% of wild type but brain ABCA1 protein levels were similar to wild type (Figure 3-15). Normal level of brain ABCA1 protein in BALB/c *Npc1*$^{-/-}$ mice was shown recently by Karten *et al.* [251]. Considering the drastic decrease of plasma HDL-cholesterol (17% of wild type) levels in liver-specific *ABCA1* knockout mice [42], normal HDL-cholesterol levels in BALB/c *Npc1*$^{-/-}$ mice despite a significant decrease in ABCA1 protein is not easy to explain. Although the biochemical and pathologic changes in *Npc1*$^{-/-}$ mice are similar to those seen in humans [252, 253], lipoprotein physiology varies considerably between rodents and humans [254]. The findings presented here suggest striking differences in the impact of NPC deficiency on HDL metabolism in mice compared with humans.

A. Liver ABCA1 protein levels



B. Brain ABCA1 protein levels

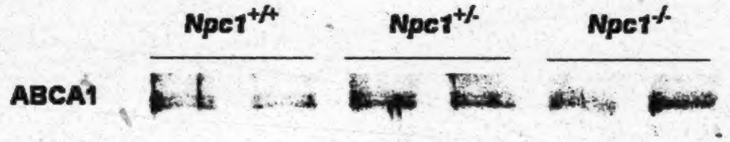


Figure 3-15. ABCA1 protein levels in 5 week old BALB/c mouse tissues. ABCA1 protein was detected by Western blotting of 100 μg of total liver **(A)** or brain **(B)** proteins with a rabbit polyclonal antibody to ABCA1. Numeric values represent the densities of ABCA1 protein bands normalized to protein disulfide isomerase (PDI) loading control, and relative to the average of ABCA1 protein levels in two *Npc1*$^{+/+}$ mouse livers. Results are representative of two separate experiments with similar results.

3.6 Conclusions

The results presented here demonstrate an additional defect in regulation of a cholesterol-dependent gene, *ABCA1*, in NPC disease. The data suggest that this dysregulation is responsible for the hypoalphalipoproteinemia seen in the majority of NPC disease patients studied. However, these data do not allow us to draw conclusions about whether the severity of clinical disease in NPC patients correlates with their level of ABCA1 dysfunction and/or HDL-cholesterol level.

The ability of a specific LXR agonist TO-901317 to upregulate *ABCA1* for the correction of lipid efflux and HDL particle formation by human NPC disease fibroblasts demonstrates that *NPC1* mutation-induced impairment of ABCA1 regulation and function can be bypassed using exogenous LXR agonists. These results have importance in understanding the interrelationships between ABCA1 and NPC1 in cell cholesterol homeostasis, and provide a potential therapeutic mechanism for correction of lipid accumulation and trafficking in NPC disease.

Further studies are required to examine ABCA1-specific role in the correction of lipid efflux and HDL formation by NPC disease cells, and to understand the role of ABCA1 in the pathogenesis of NPC disease.

CHAPTER 4:

Impaired Apolipoprotein A-I-mediated Cholesterol Efflux as a Novel Marker of Epithelioid Arterial Smooth Muscle Cells

4.1 Introduction

4.1.1 Atherosclerosis and cholesterol

Atherosclerosis and its clinical manifestations, coronary heart disease and stroke, are the leading causes of death in the world in adults [255]. Multiple processes including endothelial dysfunction, inflammation, vascular cell proliferation and matrix alteration are involved in the progression of atherosclerosis. The presence of cholesteryl ester (CE)-overloaded foam cells in the intimal layer of arteries is considered the biochemical hallmark of atherosclerosis [256, 257]. Cholesterol deposited in atherosclerotic lesions is derived from pathogenically modified forms of apolipoprotein B (apoB)-containing lipoproteins such as low density lipoprotein (LDL) and very low density lipoprotein (VLDL) [258], and therefore a high concentration of apoB-containing lipoprotein in the circulation is pro-atherogenic. On the other hand, high density lipoprotein (HDL) and its apolipoproteins are believed to protect against atherosclerosis by mediating removal and transport of cholesterol from atherosclerotic lesions to the liver for excretion into bile, in a process called reverse cholesterol transport [87, 259]. A high level of circulating HDL in human plasma is one of strongest predictors of protection against the clinical manifestations of atherosclerosis [260].

4.1.2 Arterial smooth muscle cells and cholesterol

Arterial smooth muscle cells (SMC) are found only in the medial layer of undiseased arteries. SMC appear in the intimal layer of injured arteries [261], and represent the predominant cell type in the intima as atherosclerotic lesions develop [262].

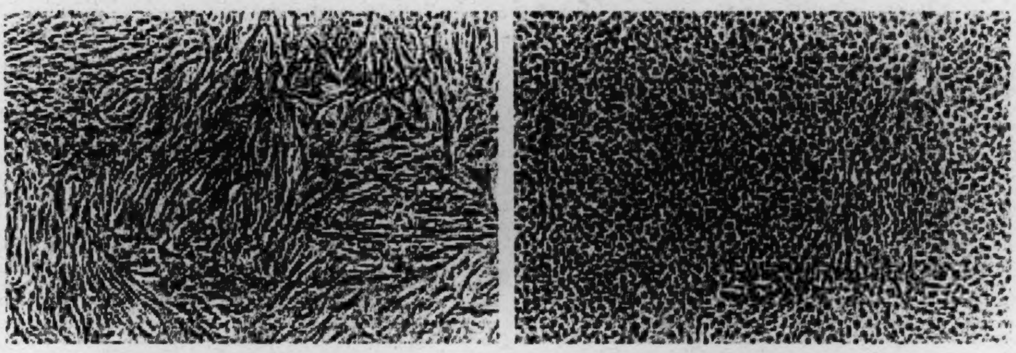
Intimal SMC are thought to originate from SMC migrating into the developing atherosclerotic lesion from the medial layer of arteries [262]. However, many studies indicate that intimal SMC may also originate from other cell types. Adventitial fibroblasts were reported to be able to express a smooth muscle marker, smooth muscle α-actin, and migrate to the intima after vascular balloon injury in rats [263]. Quail embryonic endothelial cells were reported to be able to trans-differentiate into mesenchymal cells expressing smooth muscle α-actin

[264]. Animal studies showed that intimal SMC can originate from bone marrow- and non-bone-marrow-derived circulating cells [265-268]. Smooth muscle progenitor cells have been identified in human blood [269]. These findings suggest that intimal SMC might originate from diverse cell types, but the relative contribution of each site of origin is presently unknown.

Intimal SMC produce large amounts of extracellular matrix components including collagen and proteoglycans to maintain the integrity of the injured vessel. Proteoglycans contain highly negatively charged carbohydrate chains, and associate with positively-charged arginine and lysine residues on apoB in lipoproteins infiltrated into the intima. These lipoproteins can then be modified and taken up by intimal SMC and macrophages to become foam cells [258, 270]. It is therefore believed that the accumulation of SMC and CE in the intima are key events in the pathogenesis of atherosclerosis [257, 262, 271]. It has been reported that scavenger receptors, such as class A scavenger receptor and CD36, mediate uncontrolled uptake of modified lipoproteins into intimal SMC as well as macrophages, leading to CE-enriched foam cell formation [185, 186, 272]. In addition, SMC isolated from human atherosclerotic lesions showed an increased capacity to esterify cholesterol [193]. However, it is not clear yet how intimal SMC release excess cholesterol to lipid acceptors, HDL and its apolipoproteins, in order to maintain their cholesterol homeostasis. The ability of cells to release excess cholesterol to apolipoprotein A-I (apoA-I), the most abundant apolipoprotein on HDL, and thus form HDL particles is determined by the interaction between apoA-I and ATP-binding cassette transporter A1 (ABCA1), a membrane lipid transporter upregulated by high cell cholesterol content. The presence of CE-engorged SMC in the intima leads us to hypothesize that intimal SMC are defective in apoA-I-mediated cholesterol efflux.

4.1.3 Heterogeneity of arterial smooth muscle cells

Several lines of investigation including tissue explantation and cloning from primary culture have indicated that arterial SMC are heterogeneous in morphology, growth properties, and gene expression patterns in various species including rat, cow, pig and human (reviewed in [190]). Arterial SMC have been categorized into two main morphologic subtypes, spindle and epithelioid phenotype. Their morphologic appearances are shown in Figure 4-1. Irrespective of the species, epithelioid SMC are characterized as less differentiated than spindle SMC, and exhibit greater proliferation, poor contraction, and faster migration compared to spindle SMC [190]. With their enhanced migrating and proliferating ability, epithelioid SMC are thought to migrate from the media to the intima, expand the intima, and become atherogenic SMC. In support of this scenario, epithelioid-shaped synthetic SMC are the predominant SMC subtype in the thickened intima, whereas spindle-shaped contractile SMC are typical in the normal media [191, 192, 273].



Spindle SMC **Epithelioid SMC**

Figure 4-1. Morphological aspects of two main SMC subtypes. Photomicrographs show spindle- and epithelioid-morphology rat SMC clones isolated from the thoracic aorta. The figure was obtained from M.L. Bochaton-Piallat *et al* [191] and is presented here with permission.

The accumulation of CE and the predominance of epithelioid-shaped synthetic SMC in the intima suggest that epithelioid SMC accumulate CE. In further support of the hypothesis that intimal SMC are defective in the release of cholesterol, Francis et al. [246] reported that cultured human medial (spindle) arterial SMC bind and release phospholipids and cholesterol to apoA-I, while SMC obtained from a rat pup aorta and having an epithelioid phenotype fail to bind or release lipids to apoA-I.

4.1.4 Study objectives

To test the hypothesis that intimal-phenotype epithelioid SMC are defective in apoA-I-mediated cholesterol efflux, the ability of SMC of varying phenotype to release cholesterol and phospholipids to apoA-I and to bind apoA-I are examined. To correlate apoA-I-mediated lipid efflux results with ABCA1 function, expression levels of ABCA1 are also examined.

4.2 Methods

4.2.1 Cell lines used in this study

No human atherosclerotic intima SMC line suitable for use in continuous culture studies is presently available. In order to examine whether apoA-I-mediated cholesterol efflux is defective in intimal-phenotype SMC, rat and human SMC lines characterized as epithelioid- or spindle-shaped were used. WKY12-22 and SD12d SMC lines derived from thoracic aortas of 12-day old Wistar-Kyoto rats and Sprague-Dawley rats, respectively, were characterized as epithelioid-shaped SMC, and WKY3M-22 and SD3M-23 SMC lines derived from 3-month old Wistar-Kyoto or Sprague Dawley rats were characterized as spindle-shaped SMC [274-276]. These cell lines were a kind gift from Dr. Joan Lemire, University of Washington. HITA2 SMC derived from human internal thoracic artery (a kind gift of Dr. Geoff Pickering, University of Western Ontario) were characterized as epithelioid-shaped SMC [277], and human aortic smooth muscle cells (HASMC) from American Type Culture Collection (CRL-1999) were characterized as spindle-shaped SMC.

4.2.2 Cell culture

Frozen vials of the three epithelioid-shaped SMC lines (WKY12-22, SD12d, and HITA2) and three spindle-shaped SMC lines (WKY3M-22, SD3M-23, and HASMC) were thawed and cultures re-established in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were subcultured using 0.25% trypsin-EDTA (Invitrogen, Burlington, ON) whenever they became confluent and were used between the second and tenth passage after re-establishing the culture. All the six cell lines maintained their distinct morphologic phenotypes and growth rates during these passages.

To load cells with cholesterol, confluent cell layers were rinsed twice with phosphate-buffered saline (PBS) containing 1 mg/ml BSA (PBS/BSA) and incubated for 24 h in DMEM containing 2 mg/ml BSA with 30 µg/ml cholesterol added from a 10 mg/ml stock in ethanol. To allow equilibration of added cholesterol, cell layers were rinsed twice with PBS/BSA and incubated for an additional 24 h in DMEM containing 1 mg/ml BSA (DMEM/BSA).

4.2.3 Cholesterol efflux assay

Cells were seeded on 16-mm wells, non-LDL-derived cellular cholesterol pools were radiolabeled during the last 40% of growth to confluence, and efflux assays were performed as described in Section 2.3 – 2.5.

4.2.4 Phospholipid efflux assay

Cells were seeded on 35-mm wells, choline-containing phospholipids were radiolabeled, and efflux assays were performed as described in Section 2.3 – 2.5.

4.2.5 Cholesterol esterification assay

Cells were seeded on 16-mm wells and grown to confluence in DMEM containing 10% FBS. To assess the size of the pool of cell cholesterol available for esterification by ACAT following incubation with apoA-I, cells loaded with non-lipoprotein cholesterol and equilibrated were rinsed once with PBS/BSA, incubated for 16 h in DMEM/BSA containing 0-10 µg/ml apoA-I, rinsed once with PBS, and incubated for 1 hr more with DMEM containing 9 µM [^{14}C]oleate bound to 3 µM BSA [246]. At the end of the incubation, cells were chilled on ice, rinsed twice with iced PBS/BSA and twice with iced PBS, and stored at –20 °C until lipid extraction. Cellular lipids were extracted and separated by thin layer chromatography, and the cholesteryl ester spot was obtained for determination of radioactivity as described in Section 2.5. Cell proteins were determined by the Lowry assay using BSA as standard.

4.2.6 Sterol mass analysis by gas chromatography

Cells were seeded on 60-mm dishes and grown to confluence in DMEM containing 10% FBS. The cells were loaded with non-lipoprotein cholesterol for 24 h and equilibrated for 24 h. The cells were then rinsed twice with PBS/BSA and incubated for 24 h in DMEM/BSA with or without 10 μg/ml apoA-I. At the end of the incubation, media and cells were collected for sterol mass analyses as described in Section 2.6.

4.2.7 Cellular binding of apoA-I

Cells were grown to confluence on 35-mm wells in DMEM containing10% FBS, then incubated with or without 30 μg/ml cholesterol for 24 h and equilibrated in DMEM/BSA for 24 h. Cells were then incubated for 2 h at 4 °C in DMEM/BSA containing 25 mM HEPES and 10 μg/ml ^{125}I-apoA-I ± 200 μg/ml unlabeled apoA-I [160]. Cells were rinsed 5 times with iced PBS/BSA and twice with iced PBS. Cell layers were dissolved in 0.2 N NaOH and aliquots were taken for quantification of radioactivity and protein. Cell surface ^{125}I-apoA-I bound per mg of cell protein was determined by subtracting values in the presence of the excess unlabeled apoA-I to exclude non-specifically bound ^{125}I-apoA-I.

4.2.8 Quantitative real-time PCR analysis of ABCA1

Cells were grown to confluence on 35-mm wells in DMEM containing 10% FBS, then incubated with or without 30 μg/ml cholesterol for 24 h and equilibrated in DMEM/BSA for 24 h prior to determining ABCA1 mRNA levels. Total RNA was isolated as described [278]. Two micrograms of RNA were treated with DNase I (Invitrogen, Burlington, ON) and used for first-strand cDNA synthesis. The RNA was incubated with 0.5 μg of oligo $(dT)_{12\text{-}18}$ primer (Invitrogen) at 70 °C for 10 min and then reverse-transcribed by incubating with 100 units of Superscript RNase H^- Reverse Transcriptase (Invitrogen), 1x first-strand buffer, 0.5 mM dNTP mix, 0.01 M dithiothreitol, 1 μg BSA, and 2 units of RNase Inhibitor (Invitrogen) in a 20 μl reaction volume at 45 °C for 90 min. The reaction was terminated by inactivating the polymerase at 95 °C for 3 min and then put promptly on ice. Real-Time PCR analyses were performed using the Rotor-Gene 3000 instrument and software (Corbett Research, Kirkland, PQ). DNA amplification was monitored by detecting the binding of a fluorescent dye SYBR Green I (Molecular Probes, Burlington, ON) to double-stranded DNA.

Each 25 μl reaction contained 1X PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTP mix, 1 μl of SYBR Green I diluted in 1/1000 in sterile water, 0.3 μM each primer, 0.25 unit of Platinum *Taq* DNA polymerase (Invitrogen), and template cDNA. All samples were run in triplicate and a no template control was run to determine the level of primer dimer formation. PCR parameters for both the target gene ABCA1 and a reference gene cyclophilin were an initial denature at 95 °C for 120 s followed by 40 cycles of 95 °C for 20 s, 62 °C for 15 s, and 72 °C for 15 s. Specific amplification of a single PCR product with the desired length (ABCA1, 152 bp and cyclophilin, 356 bp) was confirmed with high resolution gel electrophoresis and melting curve analysis. The relative abundance of ABCA1 mRNA in comparison to cyclophilin mRNA was calculated by the Pfaffl analysis method [279]. The primers used are as follows: ABCA1, 5'-AGT ACC CCA GCC TGG AAC TT (forward), 5'-TGG GTT TCC TTC CAT ACA GCG (reverse); cyclophilin, 5'-TCC AAA GAC AGC AGA AAA CTT TCG (forward), 5'-TCT TCT TGC TGG TCT TGC CAT TCC (reverse).

4.2.9 Western blot analysis of ABCA1

Cells were grown to confluence on 75 cm^2 flasks in DMEM containing 10% FBS, then incubated with or without 30 μg/ml cholesterol for 24 h and equilibrated in DMEM/BSA for 24 h prior to determination of ABCA1 protein levels. Crude membranes proteins were prepared by homogenizing cells on ice in 50 mM Tris-HCl buffer, pH 7.4, containing complete protease inhibitors (Roche Molecular Biochemicals, Laval, QC) and 1 mM EDTA. The nuclear fraction was removed by centrifugation for 2 min at 5,000 rpm (2,040 x g), and the supernatant was subsequently centrifuged for 30 min at 14,000 rpm (16,000 x g). The pellet was then resuspended in the homogenizing buffer with 0.45 M urea, 0.1 % Triton X-100, 0.2 % SDS and 0.05 % dithiothreitol and protein concentrations were determined. Fifty micrograms of membrane proteins were separated by 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. Immunoblotting was performed as described in Section 2.7 using a rabbit polyclonal antibody to ABCA1 (1:1000, Novus Biologicals, Littleton, CO) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000, Sigma-Aldrich, Oakville, ON).

4.2.10 Western blot analysis of apoA-I contained in media

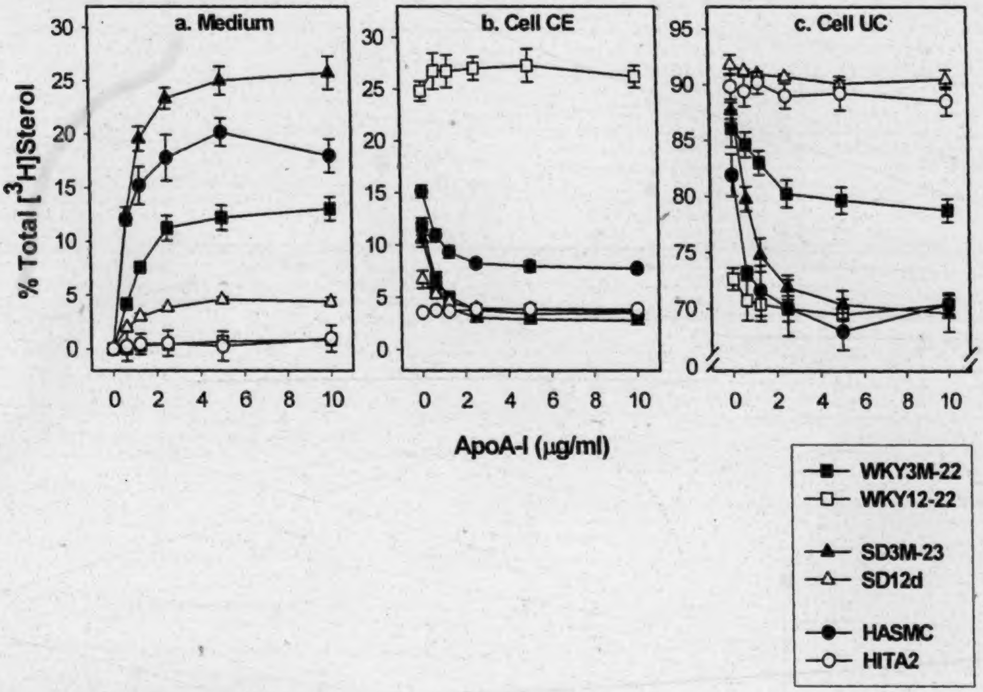
Cells were grown to confluence on 35-mm wells in DMEM containing10% FBS, then incubated with 30 µg/ml cholesterol for 24 h and equilibrated in DMEM/BSA for 24 h. Cells were then incubated for 24 h in DMEM/BSA containing 10 µg/ml apoA-I. During the 24 h incubation period, 200 µl of media were collected at 0, 12, and 24 h time points to determine apoA-I levels contained in media. Forty microliters of media were analyzed by 15% SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. Immunoblotting was performed as described in Section 2.7 using a rabbit polyclonal antibody to apoA-I (1:10,000, Calbiochem, San Diego, CA) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000, Sigma-Aldrich, Oakville, ON).

4.3 Results

4.3.1 ApoA-I-mediated cholesterol efflux is impaired in epithelioid morphology SMC

ApoA-I removes cellular cholesterol by interacting with ABCA1 upregulated in response to increased cell cholesterol content. I found that this active cholesterol removal pathway is nearly absent or markedly low in all three epithelioid-morphology SMC lines (open symbols) examined, whereas all three spindle-morphology SMC lines (filled symbols) actively release cholesterol to apoA-I in a dose-dependent and saturable manner (Figure 4-2Aa). This dramatic difference between SMC subtypes in apoA-I-mediated cholesterol efflux is very well correlated with the expression levels of ABCA1 protein (Figure 4-2B). Two epithelioid SMC lines (WKY12-22 and HITA2) express a trace amount ABCA1 protein at basal level but show no significant increase in response to cholesterol loading, which correlates with the near absence of apoA-I-mediated cholesterol efflux. Although another epithelioid cell line, SD12d, expressed ABCA1 to a similar extent as spindle SMC from the same species (SD3M-23) at basal level, cholesterol-stimulated increase in ABCA1 was seen only in the SD3M-23 cell line (Figure 4-2B). The basal level of ABCA1 in SD12d cells explains the small extent of cholesterol efflux to apoA-I. The near absence of apoA-I-mediated cholesterol release to medium from WKY12-22 and HITA2 cells is reflected in no significant change in radiolabeled cellular cholesteryl ester (CE) (Figure 4-2Ab) and unesterified cholesterol (UC) (Figure 4-2Ac) in these cells. The small amount of cholesterol efflux from SD12d cells is reflected in a corresponding decrease in cellular CE label. Impaired cholesterol efflux from WKY12-22 cells was accompanied by accumulation of cellular CE label (Figure 4-2Ab), in contrast to the accumulation of UC label in SD12d and HITA2 cells (Figure 4-2Ac). The finding of CE accumulation in WKY12-22 cells is in accordance with previous observations that human atherosclerotic lesions accumulate CE, and that human intimal SMC have an increased capacity to esterify cholesterol [193]. For this reason, it appears that among the three epithelioid SMC lines tested, the

A. Cholesterol efflux



B. ABCA1 protein levels

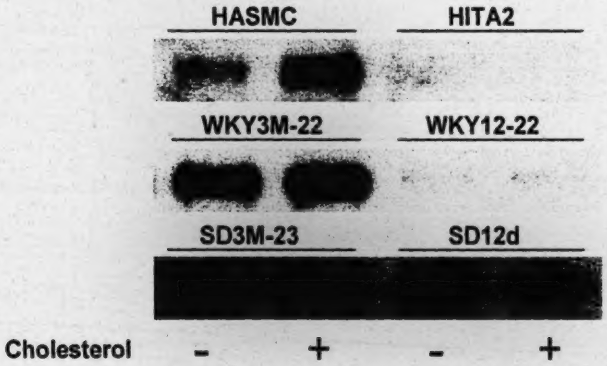

HASMC
HITA2
WKY3M-22
WKY12-22
SD3M-23
SD12d
Cholesterol
-
+
-
+

Figure 4-2. ApoA-I-mediated cholesterol efflux from arterial SMC and the expression levels of ABCA1. (A) Cells were labeled with [^{3}H]cholesterol during growth, loaded with unlabeled cholesterol for 24 h, equilibrated for 24 h, and incubated with the indicated concentration of apoA-I for 24 h. At the end of the

incubation the medium was removed and cell cholesteryl ester (CE) and unesterified cholesterol (UC) in cells and media were analyzed for [^{3}H]cholesterol. Results were expressed as percent of total cell plus medium [^{3}H]sterol in the medium (a), cell CE (b), and cell UC (c) following subtraction of efflux to medium containing 1 mg/ml BSA alone. Results for WKY cells represent averages ± S.D. of three separate experiments performed in quadruplicate. Results for SD and human cells show the mean ± S.D. of quadruplicate determinations and are representative of two separate experiments with similar results. **(B)** Cells were grown to confluence in DMEM/10% FBS, then incubated with or without 30 µg/ml cholesterol for 24 h and equilibrated in DMEM/BSA for 24 h prior to determination of ABCA1 protein levels. ABCA1 protein was detected by Western blotting of 50 µg of cellular membrane protein with a rabbit polyclonal antibody to ABCA1. Results are representative of three or more separate experiments with similar results.

WKY12-22 SMC line represents the best model of human atherosclerotic intima SMC.

The absence of depletion of CE accumulated in WKY12-22 SMC in response to apoA-I provides further evidence for the absence of ABCA1 function, based on the observation that ABCA1 releases regulatory pools of cholesterol to apoA-I that would be otherwise esterified by acyl CoA:cholesterol acyltransferase (ACAT) [88]. To confirm this, I assessed the ability of WKY cells to deplete cholesterol pools available for esterification by ACAT. Addition of apoA-I to WKY12-22 cells did not affect their subsequent ability to esterify cholesterol, whereas WKY3M-22 cells were depleted of cholesterol pools available for esterification in an apoA-I dose dependent and saturable manner (Figure 4-3).

These results suggest that all epithelioid SMC lines studied here are impaired in apoA-I-mediated cholesterol efflux, but that the WKY12-22 cell line most closely resembles human intimal SMC based on the accumulation of cholesteryl ester. For this reason, the WKY SMC lines were chosen for my subsequent studies.

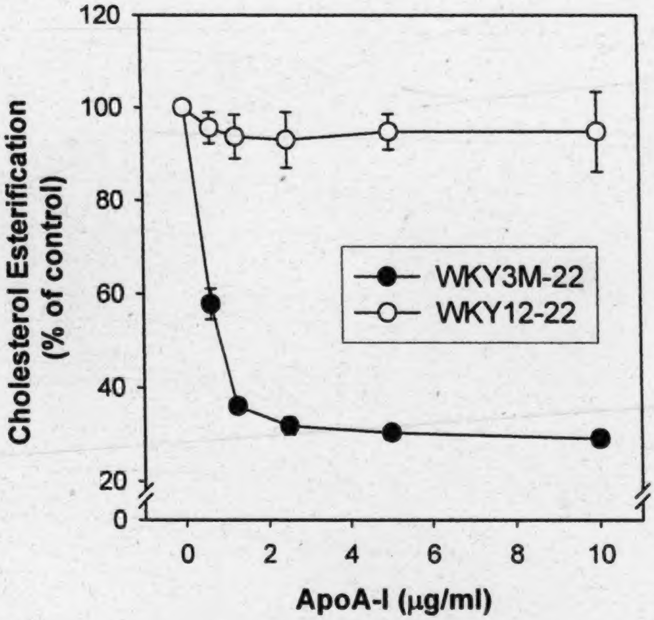


Figure 4-3. ApoA-I-mediated depletion of cell cholesterol available for esterification in WKY SMC. Confluent cells were loaded with 30 μg/ml cholesterol for 24 h, and then equilibrated in DMEM/BSA for 24 h. Cells were then incubated with DMEM/BSA plus the indicated concentration of apoA-I for 16 h. After washing, cells were incubated for 1 h with [^{14}C]oleate to assess formation of cellular cholesteryl [^{14}C]oleate. Results are the mean ± S.D. of quadruplicate determinations, expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl ester per milligram of cell protein per hour in cells treated with DMEM/BSA alone (control), and are representative of three separate experiments with similar results.

4.3.2 WKY12-22 SMC are impaired in cholesterol mass efflux

Although removal of radiolabeled cholesterol is the most commonly used method for assessing cholesterol efflux from cultured cells, a potential problem with this method is that it may not reflect similar changes in cell cholesterol mass. To quantitate net cholesterol movement directly, we measured sterol mass using gas chromatography as described in Section 4.2.5. WKY3M-22 cells showed an approximate doubling of cholesterol mass efflux to DMEM/BSA medium containing 10 µg/ml apoA-I when compared to DMEM/BSA medium alone. WKY12-22 cells released a minimal amount of cholesterol to DMEM/BSA medium, and showed no increase in cholesterol mass efflux to apoA-I-containing medium (Figure 4-4A). WKY3M-22 cells were depleted of ~55% of CE mass during the incubation with apoA-I, whereas WKY12-22 cells showed no depletion of CE mass even though they accumulated markedly more CE compared to WKY3M-22 cells (Figure 4-4B). Although there was no significant change in cellular UC levels in both cell lines, there was a trend to decreased UC in WKY3M-22 in the presence of apoA-I (Figure 4-4C). These results confirm that WKY3M-22 cells actively release excess cholesterol mass to apoA-I, whereas WKY12-22 cells are impaired in apoA-I-mediated cholesterol efflux.

Cholesterol efflux to the DMEM/BSA medium without apoA-I is thought to occur through passive diffusion of cholesterol from the cell surface to the medium. The markedly low passive diffusion from WKY12-22 cells compared to WKY3M-22 cells (Figure 4-4A) might suggest that the concentration or localization of cholesterol in the surface of WKY12-22 cells is not optimal for passive diffusion.

To see if the impaired apoA-I-mediated cholesterol efflux from WKY12-22 cells was due to any degradation of apoA-I during the 24 hr incubation with cells, I examined apoA-I levels in media after 0, 12, and 24 hr incubations with cells. The amount of apoA-I contained in both WKY3M-22 and WKY12-22 cell-conditioned medium was not altered throughout the incubation period (Figure 4-5), indicating that degradation of apoA-I is not the reason for impaired cholesterol efflux from WKY12-22 cells.

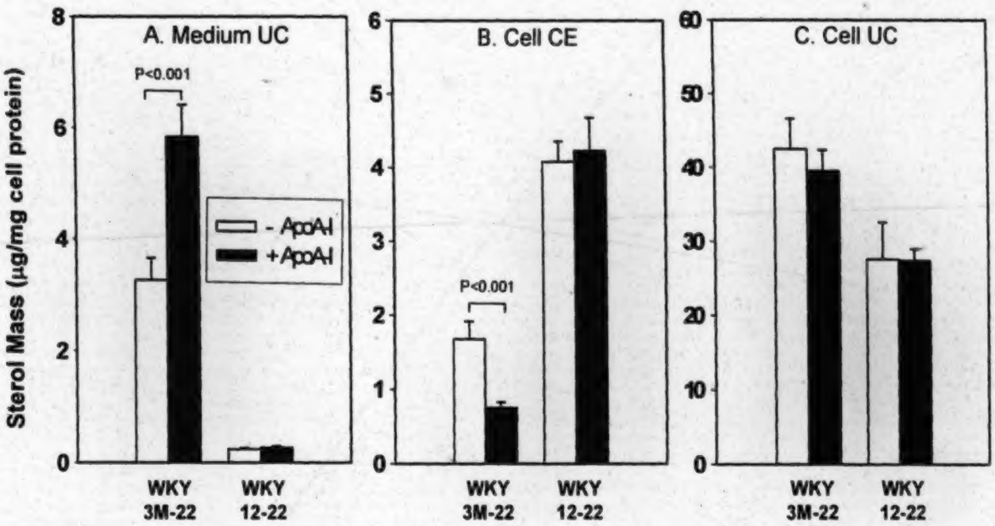


Figure 4-4. ApoA-I-mediated efflux of cholesterol mass from WKY SMC. Confluent cells were loaded with 30 µg/ml cholesterol for 24 h, and then equilibrated in DMEM/BSA for 24 h. Cells were then incubated for 24 h in DMEM/BSA with or without 10 µg/ml apoA-I. At the end of the incubation, media and cells were collected and total lipids were extracted. Unesterified cholesterol (UC) mass in the medium **(A)**, cellular cholesteryl ester (CE) mass **(B)**, and cellular UC mass **(C)** were determined by gas chromatography as described in Section 4.2.5. Results represent averages ± S.D. of two separate experiments performed in quadruplicate.

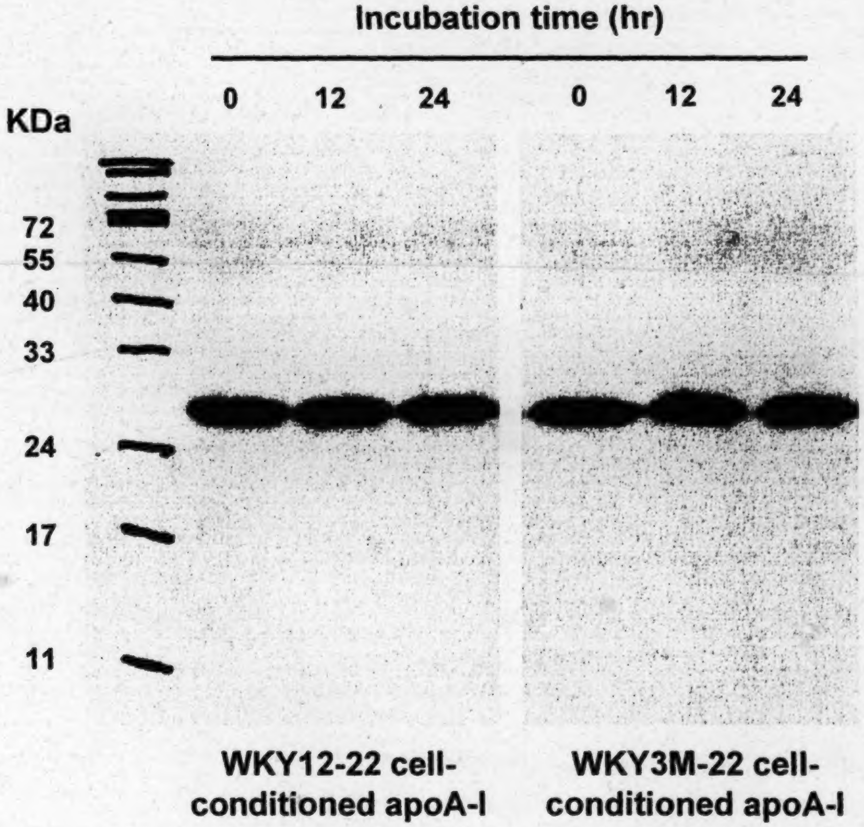

Incubation time (hr)
0
12
24
0
12
24
KDa
72
55
40
33
24
17
11
WKY12-22 cell-conditioned apoA-I
WKY3M-22 cell-conditioned apoA-I

Figure 4-5. ApoA-I levels contained in media during incubation with WKY SMC. Confluent cells were loaded with 30 μg/ml cholesterol for 24 h, and then equilibrated in DMEM/BSA for 24 h. Cells were then incubated for 24 h in DMEM/BSA containing 10 μg/ml apoA-I. During the 24 h incubation period, aliquots of media were collected at 0, 12, and 24 h time points to determine apoA-I levels contained in media by Western blots. Results are representative of two separate experiments with similar results.

4.3.3 Epithelioid SMC are impaired in apoA-I-mediated choline-containing phospholipid efflux

The mechanism of apoA-I-mediated cholesterol efflux is still in debate, however, phospholipidation of apoA-I by ABCA1 is believed to be required simultaneously or prior to cholesterol efflux, as discussed in Section 1.2.4.3. Phosphatidylcholine (PC) is the major phospholipid (PL) substrate transferred by ABCA1 [87]. [^{3}H]choline-labeled WKY12-22 cells showed only a minimal amount of [^{3}H]PC and [^{3}H]sphingomyelin (SM) release to apoA-I, in contrast to active efflux of both phospholipids from WKY3M-22 cells in a dose-dependent and saturable manner (Figure 4-6). Similar experiments with HITA2 and HASMC cells also showed the same results: near absence of [^{3}H]PC and [^{3}H]SM efflux from the epithelioid HITA2 cells, in contrast to active efflux from spindle-shaped HASMC (Figure 4-7). These results suggest that epithelioid SMC are impaired in choline-containing PL efflux due to defective ABCA1 action.

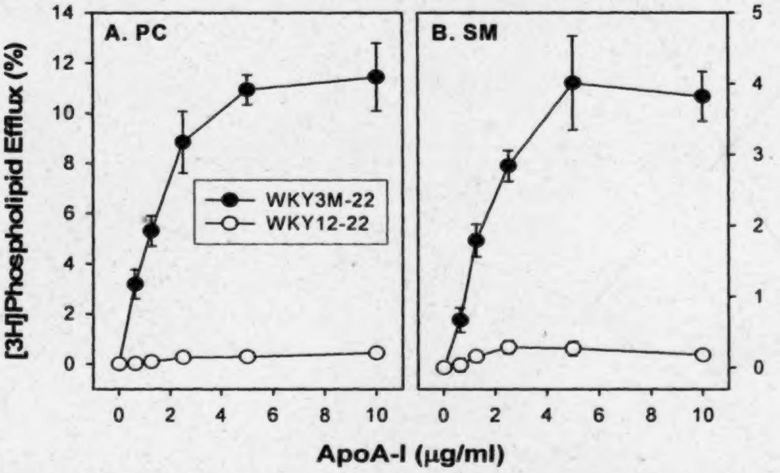


Figure 4-6. ApoA-I-mediated efflux of choline-containing phospholipids from WKY SMC. Cells loaded with cholesterol and radiolabeled with [^{3}H]choline chloride for 24 h were incubated with the indicated concentration of apoA-I for 24 h to determine efflux of phosphatidyl[^{3}H]choline (PC) **(A)** and [^{3}H]sphingomyelin (SM) **(B)**. Results are averages ± S.D. of two separate experiments performed in quadruplicate, expressed as the percentage of total cellular plus medium [^{3}H]counts for PC or SM in the medium following subtraction of efflux to medium containing 1 mg/ml BSA alone.

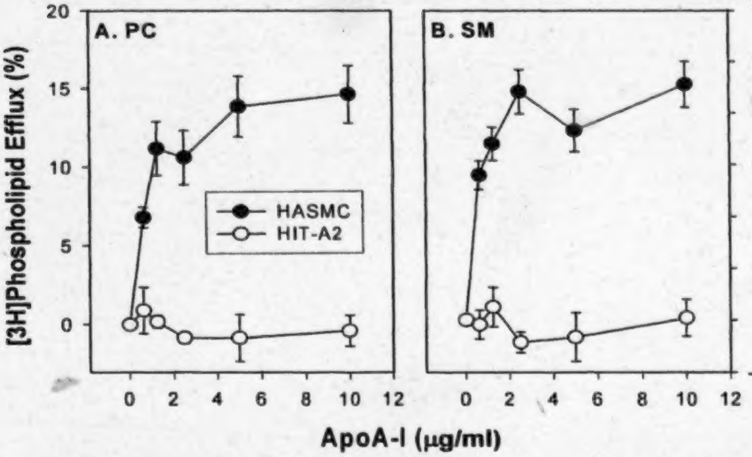


Figure 4-7. ApoA-I-mediated efflux of choline-containing phospholipids from human arterial SMC. Experimental procedures and analysis of data are as in Figure 4-5 legend. Results are averages ± S.D. of one experiment performed in quadruplicate.

4.3.4 ABCA1 regulation in WKY12-22 SMC is impaired at the level of mRNA

Although ABCA1 transcription is strongly upregulated in response to increasing cell cholesterol content, ABCA1 protein levels are also regulated at post-translational steps [87]. To find at which step regulation of ABCA1 in WKY12-22 cells is impaired, we measured mRNA abundance by Real-Time PCR. WKY3M-22 cells had a high basal level of ABCA1 mRNA and significantly increased ABCA1 transcription in response to cholesterol loading, whereas WKY12-22 cells had a markedly low basal level of ABCA1 mRNA and showed no significant increase after cholesterol loading (Figure 4-8). ABCA1 mRNA levels were consistent with the protein levels shown in Figure 4-2B, indicating that ABCA1 regulation in WKY12-22 cells is impaired at a step of transcription or possibly mRNA stabilization.

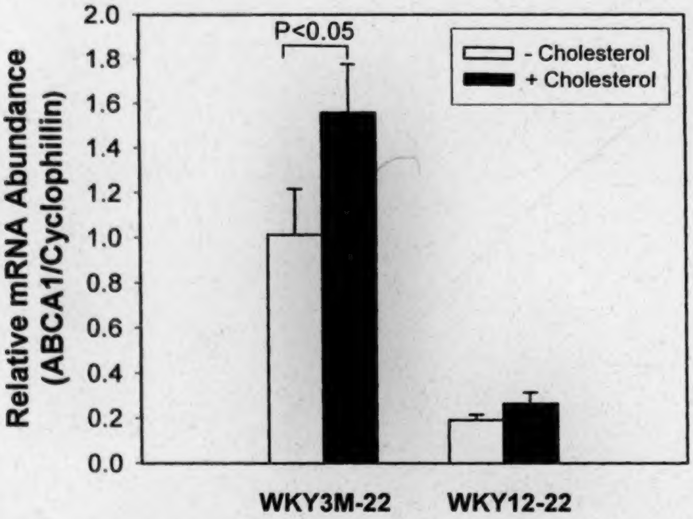


Figure 4-8. Quantification of ABCA1 mRNA levels in WKY SMC. Confluent cells were incubated with or without 30 µg/ml cholesterol for 24 h, and then equilibrated in DMEM/BSA for 24 h prior to determination of ABCA1 mRNA levels by Real-Time PCR. Levels of ABCA1 mRNA were normalized to cyclophilin mRNA levels. Results are averages ± S.D. of three separate experiments and relative to the ratio of ABCA1/cyclophilin mRNA in non-cholesterol loaded WKY3M-22 cells.

4.3.5 WKY12-22 SMC have low binding capacity to apoA-I

Direct binding of apoA-I to ABCA1 and/or a cellular association of apoA-I with lipid domains formed by ABCA1 are thought to be necessary for apoA-I-mediated lipid efflux [87, 114]. To assess whether binding capacity of WKY SMC to apoA-I is correlated with ABCA1 levels and lipid efflux results, we measured cellular binding capacity to apoA-I at 4 °C. WKY12-22 cells showed a markedly low binding capacity in both basal and cholesterol-loaded conditions compared to WKY3M-22 cells (Figure 4-9). These results suggest that a defective interaction between WKY12-22 cells and apoA-I leads to impaired lipid efflux from WKY12-22 cells.

The apoA-I binding results are somewhat inconsistent with ABCA1 expression levels shown in Figure 4-2B and Figure 4-8. Cholesterol-loaded WKY12-22 cells showed a small but statistically significant increase in their apoA-I binding capacity despite no significant increase in ABCA1 levels, suggesting that interaction between cells and apoA-I might be facilitated not only by ABCA1 but by additional factors.

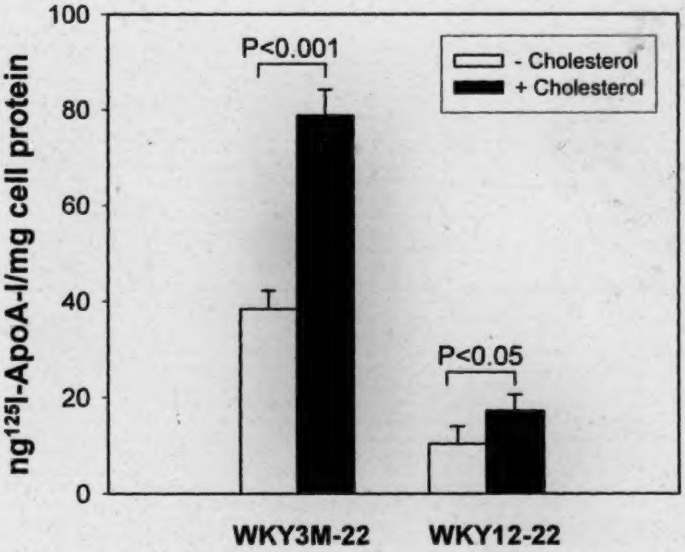


Figure 4-9. Binding of ^{125}I-apoA-I to WKY SMC. Confluent cells were incubated with or without 30 μg/ml cholesterol for 24 h, and then equilibrated in DMEM/BSA for 24 h. Cells were then incubated with 10 μg/ml ^{125}I-apoA-I ± 200 μg/ml unlabeled apoA-I for 2 h at 4 °C. After extensive rinsing, cells were assessed for radioactivity. Cellular ^{125}I-apoA bound per mg of cell protein was determined by subtracting values in the presence of the unlabeled apoA-I. Results are the mean ± S.D. of quadruplicate determinations and are representative of three separate experiments with similar results.

4.4 Discussion and conclusions

It has been reported that SMC derived from the aortas of several animal species, including rat, monkey, rabbit, and pig are resistant to apoA-I-mediated cholesterol efflux [280-282]. Our laboratory previously showed that SMC derived from human thoracic aorta efficiently bind and release choline-containing PL and cholesterol to apoA-I [246]. Here I show that the responsiveness of arterial SMC to apoA-I is dependent not on species but on SMC subtype. Considering the heterogeneity of arterial SMC and the predominance of epithelioid-morphology SMC in atherosclerotic lesions, it is very important to characterize subpopulations of SMC rather than mixed, whole cell populations. Our finding that epithelioid, intimal-phenotype SMC are impaired in apoA-I-mediated lipid efflux suggests a crucial reason for CE accumulation in atherosclerotic lesion SMC.

Although all the three epithelioid SMC lines studied here were defective in apoA-I-mediated cholesterol efflux, CE accumulation, the biochemical hallmark of foam cell formation in atherosclerotic lesions, was observed only in WKY12-22 SMC. These results indicate that epithelioid SMC lines are not all the same in terms of cholesterol metabolism. This seems to be true even in SMC lines derived from the same species, as shown by the differences between WKY and SD rat SMC lines. The reason for the different cholesterol metabolism between epithelioid SMC lines is unclear, but based on the accumulation of cholesteryl ester, we considered the WKY12-22 SMC to be the best model of intimal epithelioid SMC present in human atherosclerotic lesions. Moreover, several distinct gene-expression patterns shown in WKY12-22 SMC were also observed in rat intimal SMC or during intimal thickening, including increased expression of monooxygenase, growth factors, transcription factors, and extracellular matrix molecules [275, 283, 284]. At this time, heterogeneity of SMC has been classified mainly based on morphologic differences, however, our finding that cholesterol metabolism is different even in SMC of similar morphology indicates that more careful characterization and classification of SMC are required.

Biochemically, epithelioid SMC are characterized by suppressed expression of contractile proteins, such as α-smooth muscle actin and smooth muscle myosin, compared to spindle SMC. However, the correlation between these contractile proteins and the development of atherosclerosis has not been elucidated. Numerous other proteins and/or mRNA expressed in a phenotype-specific manner have been identified. Some of the well characterized markers for epithelioid SMC include platelet-derived growth factor B chain (rat) [285, 286], osteopontin (rat and human) [275, 287-289], cellular retinol-binding protein-1 (rat) [290], zonula occludens 2 (rat) [275], versican (rat) [274], extracellular signal-regulated kinase (rat and cow) [276, 291], and cytokeratin 18 (mouse and human) [292, 293]. However, none of these markers have been conclusively proven to be relevant to the pathogenesis of atherosclerosis. It is critical to discover new markers providing insight into the pathogenesis of the human disease. Here I propose ABCA1 expression as a new marker of proatherogenic intimal SMC. Impaired apoA-I-mediated lipid efflux from epithelioid SMC and the resulting accumulation of CE in atherosclerotic lesions have a direct correlation and relevance to the pathogenesis of atherosclerosis. Further studies to obtain evidence of low ABCA1 expression in human intimal SMC are required to strengthen this proposal.

Rat epithelioid SMC have been reported to be more sensitive to apoptosis induced by reactive oxygen species, retinoic acid, and antimitotic drugs, when compared with spindle SMC [294, 295]. It has been known that ABCA1 promotes engulfment of apoptotic cells by exposing phosphatidylserine on the outer leaflet of the plasma membrane [296]. These findings suggest that the markedly low ABCA1 levels in epithelioid SMC may interrupt normal phagocytosis by macrophages in atherosclerotic lesions, leading to necrotic cell death and resulting in inflammatory damage and destabilization of atherosclerotic lesions. Impairment in ABCA1 regulation may therefore provide another reason for the progression of atherosclerotic disease and plaque rupture.

Impaired ABCA1 regulation in epithelioid SMC despite accumulation of CE suggests that epithelioid SMC might be defective in generation of oxysterols. Oxysterols are generated normally in cells overloaded with cholesterol, and are the most potent physiological ligands activating the nuclear receptor liver X receptor (LXR). LXR activates several target genes involved in cholesterol efflux, including ABCA1, ABCG1 and ABCG4 [144, 232, 297]. To date, ABCA1 is believed to mediate lipid efflux to lipid-free/poor apoA-I, while ABCG1 and ABCG4 have been proposed to facilitate cholesterol efflux to HDL particles [34, 60], It will therefore be intriguing to examine whether ABCG1 and ABCG4 are expressed, and whether their regulation is also impaired in epithelioid SMC. If ABCG1 and ABCG4 are expressed in SMC and facilitate cholesterol efflux to HDL, epithelioid SMC might be also impaired in HDL-mediated cholesterol efflux. In support of this speculation, it has been reported that de-differentiation of rat aortic SMC in cell culture is associated with decreased HDL-mediated cholesterol efflux [298], and that the ability of rabbit intimal SMC to release cholesterol to HDL was decreased compared to medial SMC [299]. Although these studies used whole SMC populations rather than defined subpopulations, the results support the concept that epithelioid SMC might be defective in generation of the oxysterols necessary to promote genes involved in cholesterol efflux.

In conclusion, I have found that spindle morphology SMC have intact apoA-I binding and ABCA1-mediated lipidation of apoA-I, whereas intimal-phenotype epithelioid SMC have impaired ABCA1 expression, apoA-I binding, and apoA-I-mediated lipid efflux. Impaired ABCA1 expression and apoA-I-mediated lipid efflux from epithelioid SMC may contribute to the accumulation of excess cholesterol in atherosclerotic lesions.

CHAPTER 5:

Increased ABCA1 expression fails to correct apolipoprotein A-I-mediated lipid efflux in intimal-phenotype epithelioid smooth muscle cells

5.1 Introduction

The role of smooth muscle cells (SMC) in the pathogenesis of atherosclerosis can be divided into five key events: (1) appearance and proliferation of epithelioid-shaped synthetic SMC in the intima in response to arterial injury; (2) production of large amounts of extracellular matrix by epithelioid-shaped synthetic SMC; (3) accumulation of lipid within intimal SMC as well as macrophages to form foam cells; (4) foam cell death and growth of a necrotic lipid core; and (5) rupture of the fibrous cap comprised of extracellular matrix and epithelioid-shaped synthetic SMC [190, 257, 262, 270]. The 1st and 2nd events are thought to be physiological repair processes for injured arteries. The latter 3 events are thought to be undesirable pathogenic processes that are strongly affected by lipoprotein metabolism. Apolipoprotein B (apoB)-containing lipoproteins are sources of cholesterol for foam cell formation, whereas high density lipoproteins (HDL) and their apolipoproteins decrease foam cell formation by mediating removal of excess cholesterol from intimal cells. It is therefore crucial to reduce levels of circulating apoB-containing lipoproteins and to increase HDL formation to prevent and treat atherosclerosis.

In Chapter 4, I showed that spindle morphology SMC, the typical SMC subtype in the medial layer of arteries, are active in apolipoprotein A-I (apoA-I)-mediated lipid efflux to form HDL particles, whereas epithelioid morphology SMC, the predominant SMC subtype in atherosclerotic artery intima, are impaired in apoA-I-mediated lipid efflux. Considering the presence of cholesteryl ester (CE)-enriched foam cells in the arterial intima, and previous findings that intimal SMC, like intimal macrophages, also show excess CE accumulation [193], these results have provided a clue to understand a pathogenic mechanism of CE accumulation in intimal SMC. The epithelioid SMC model chosen in Chapter 4, WKY12-22 cells, showed impaired regulation of ATP-binding cassette transporter A1 (ABCA1) at the level of mRNA in response to increased cell cholesterol content. Since ABCA1-dependent lipidation of apoA-I is believed to be the rate-limiting step in HDL particle formation [239, 300], the impaired apoA-I-mediated lipid efflux from WKY12-22 cells was ascribed to impaired regulation of ABCA1. It is therefore important to know whether the *ABCA1* gene in WKY12-22 cells is functional at all, and whether upregulation of ABCA1 expression in WKY12-22 cells corrects apoA-I-mediated lipid efflux in these intimal-phenotype SMC.

5.1.1 Study objectives

To determine whether the *ABCA1* gene in WKY12-22 cells is functional, ABCA1 expression levels and apoA-I-mediated cholesterol efflux are examined after incubating cells with exogenous liver X receptor (LXR) and/or retinoid X receptor (RXR) ligands. In addition, the ability of WKY12-22 cells transfected with ABCA1 cDNA to correct apoA-I-mediated cholesterol efflux is examined. Alterations in apoA-I binding in response to ABCA1 transfection are also examined.

5.2 Methods

5.2.1 Cell culture

WKY12-22 and WKY3M-22 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and were used between the second and tenth passage as described in Section 4.2.2. Cholesterol loading and equilibration procedures are as in Section 4.2.2.

5.2.2 Induction of ABCA1 expression and measurement of crude membrane ABCA1 protein levels

Cells were grown to confluence in 75 cm^2 flasks in DMEM containing 10% FBS. To induce ABCA1 expression, cells were incubated for 24 h in DMEM/BSA containing 10 μM 22(R)-hydroxycholesterol (Steraloids Inc., Newport, RI), 10 μM 9-cis-retinoic acid (Biomol, Plymouth Meeting, PA), 10 μM 22(R)-hydroxycholesterol plus 10 μM 9-cis-retinoic acid, 0.3 mM 8-Br-cAMP (Sigma-Aldrich, Oakville, ON) or 10 μM TO-901317 (Sigma-Aldrich, Oakville, ON). The concentration of ABCA1 inducers and incubation time were chosen based on previous reports [223, 236]. At the end of the incubation, the media were removed and the cells were harvested on ice. Preparation of crude cell membrane proteins and measurement of crude cell membrane ABCA1 levels by Western blot were performed described in Section 4.2.9.

5.2.3 Cholesterol esterification assay

Cells grown to confluence on 16-mm wells in DMEM containing 10% FBS were loaded with cholesterol, equilibrated, rinsed once with PBS/BSA, and incubated for 16 h in DMEM/BSA with the indicated concentration of apoA-I. In some experiments, TO-901317 was added during the periods of equilibration and apoA-I treatment. Cells were then rinsed once with PBS and incubated for 1 h with DMEM containing 9 μM [^{14}C]oleate bound to 3μM BSA to determine the size of the remaining cholesterol pool available for esterification by ACAT, as described in Section 4.2.5.

5.2.4 Cholesterol efflux assay and parallel measurement of total and cell surface ABCA1

Cells were grown to ~ 60% confluence on 16-mm wells and radiolabeled during the last 40% of growth by addition of 0.3 µCi/ml [^{3}H]cholesterol (NEN Life Science Products, Boston, MA) in DMEM containing 10% FBS. The confluent cells were then loaded with non-lipoprotein cholesterol, equilibrated, rinsed twice with PBS/BSA, and incubated for 24 h in DMEM/BSA containing the indicated concentration of apoA-I. In some conditions, 10 µM TO-901317 was added during the periods of equilibration and apoA-I treatment. At the end of the incubation, the media and cells were collected to determine radioactivity present in the medium or in cellular cholesterol and cholesteryl esters, as described in Section 2.5

In parallel incubations, cells seeded on 100-mm dishes and treated like those in the cholesterol efflux studies but without [^{3}H]cholesterol labeling or apoA-I incubation were chilled on ice, and cell surface proteins were biotinylated using a cell surface protein biotinylation and purification kit (Pierce, Rockford, IL) according to the manufacturer's protocol. The biotinylated samples were lysed in RIPA buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 4 mM EDTA] containing complete protease inhibitors (Roche Molecular Biochemicals, Laval, QC). Cell protein concentration was determined using BSA as standard and 100 µg of protein were run on 7.5% SDS-PAGE to determine total ABCA1 levels by Western blot as described in Section 4.2.9. The blots were reprobed with anti-protein disulfide isomerase antibody (StressGen Biotechnologies, Victoria, Canada) to verify equal protein loading. To determine cell surface ABCA1 levels, biotinylated proteins were isolated from 2 mg of total cell proteins according to the manufacturer's protocol, and 50 µl of isolated samples were run on 7.5% SDS-PAGE. ABCA1 protein levels were determined by Western blot and the blots were reprobed with anti-heat shock protein 90 (HSP90) antibody (Sigma-Aldrich, Oakville, ON) to verify no cytosolic protein in the purified samples, and we found no HSP90 signal from the purified cell surface protein, whereas HSP90 signal was detected from the blots of total cell protein.

5.2.5 Transfection of ABCA1 and cholesterol efflux assay

Cells were grown to ~ 60% confluence on 35-mm wells and radiolabeled for 24 h by addition of 0.3 μCi/ml [^{3}H]cholesterol in DMEM containing 10% FBS. At the end of the radiolabeling, the cells reached ~ 90% confluence and were transfected for 24 h with full-length murine ABCA1 cDNA cloned in a pcDNA3.1 vector that is strongly transcribed in mammalian cells via a cytomegalovirus promoter (a generous gift from Dr. Alan R. Tall, Columbia University, NY), or with empty vector, using Lipofectamine 2000 (Invitrogen, Burlington, ON). The cells were rinsed three times with PBS/BSA and incubated in DMEM/BSA containing 10 μg/ml apoA-I for 4 h because ABCA1 levels were higher after 4 h incubation than after 24 h incubation. At the end of the incubation, the media were collected and counted for radioactivity as described in Section 2.5. The cells were rinsed twice with iced PBS/BSA and twice with iced PBS, harvested, and homogenized in RIPA buffer containing complete protease inhibitors. Radioactivity remaining in cells was counted with aliquots of the homogenates. To measure ABCA1 expression levels, cell homogenates obtained following the same experimental conditions (n=4) were pooled. After determination of protein concentration using BSA as standard, 100 μg of protein was subjected to 7.5% SDS-PAGE and Western blots were performed as described in Section 4.2.8.

In parallel, cells seeded on 100-mm dishes were treated and transfected under conditions equivalent to the cholesterol efflux studies above. Total and cell surface proteins were isolated and ABCA1 levels were determined as described in Section 5.2.4. Total cell proteins were also used to detect phosphorylated ABCA1 by protein kinase A (PKA) [159]. One hundred micrograms of total cell protein were subjected to 7.5% SDS-PAGE and Western blots were performed using anti-phospho-(Ser/Thr) PKA substrate antibody (Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocol. This antibody is specific for protein containing a phospho-Ser/Thr residue with Arg at the -3 position and does not recognize nonphosphorylated PKA substrates.

5.2.6 Assessment of cholesteryl ester hydrolysis

Cells were grown to confluence on 16-mm wells in DMEM containing 10% FBS, rinsed twice with PBS/BSA, and incubated with DMEM containing 30 µg/ml cholesterol, 9 µM [^{14}C]oleate and 5 mg/ml BSA for 24 h. The cells were rinsed twice with PBS/BSA, equilibrated in DMEM containing 9 µM [^{14}C]oleate and 5 mg/ml BSA for 24 h, rinsed three times with PBS/BSA, and incubated in DMEM/BSA containing 2 µg/ml of acyl CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz 58-035 (a generous gift from Sandoz Pharmaceuticals) with or without 10 µg/ml apoA-I up to 48 h [198]. After the indicated intervals, the media were removed and cellular lipids were analyzed to determine radioactivity associated with cholesteryl esters as described in Section 2.5

5.2.7 Transfection of ABCA1 and cellular binding of ApoA-I

Cells were grown to ~70% confluence on 35-mm wells in DMEM containing10% FBS and transfected for 24 h with ABCA1 construct or empty vector as described in Section 5.2.5. After reaching confluence, the cells were rinsed twice with PBS/BSA, and incubated for 2 h at 4 °C in DMEM/BSA containing 25 mM HEPES and 10 µg/ml ^{125}I-apoA-I ± 200 µg/ml unlabeled apoA-I [160]. The cells were rinsed five times with iced PBS/BSA and twice with iced PBS. The cells were dissolved in 0.2 N NaOH and aliquots were taken to determine radioactivity associated with cells and cell protein concentration. Cell surface ^{125}I-apoA-I bound per mg of cell protein was determined by subtracting values in the presence of the unlabeled apoA-I to exclude non-specifically bound ^{125}I-apoA-I.

In parallel, cells were transfected and treated equivalent to the binding studies above, harvested, and pooled (n=3) to determine crude membrane ABCA1 levels by Western blots as described in Section 4.2.8.

5.3 Results

5.3.1 ABCA1 gene expression is normally regulated in WKY12-22 SMC

Transcription of ABCA1 is strongly induced through activation of the nuclear receptor LXR and its obligate heterodimer partner RXR, or via an LXR/RXR-independent mechanism by cyclic AMP (cAMP) [87]. LXR and RXR are activated by the binding of their ligands oxysterols and retinoic acid, respectively [138]. Either LXR or RXR ligand binding activates transcription of ABCA1, and the binding of both ligands shows an additive or synergistic activation [136].

To test whether the impaired regulation of ABCA1 mRNA in WKY 12-22 SMC in response to increased cell cholesterol content is due to a defective responsiveness of the *ABCA1* gene to its inducers, cells were treated with exogenous LXR/RXR ligands. Treatment of cells with 10 μM 22(R)-hydroxycholesterol, 10 μM 9-cis-retinoic acid, or 10 μM LXR agonist TO-901317 markedly increased cellular ABCA1 protein levels in both WKY12-22 and WKY3M-22 cells (Figure 5-1). Combined addition of 10 μM 22(R)-hydroxycholesterol plus 10 μM 9-cis-retinoic acid showed an increased effect on ABCA1 upregulation compared with either agonist alone in both cell lines (Figure 5-1). Treatment of cells with 0.3 mM 8-Br-cAMP showed no significant effect on ABCA1 protein levels in either cell line (Figure 5-1). These results indicate that the *ABCA1* gene in both WKY SMC lines is normally regulated by exogenously added LXR/RXR ligands, and that the cAMP-dependent mechanism for ABCA1 regulation seems to be absent in these WKY SMC lines.

No effect of cAMP on ABCA1 expression has also been reported in several other cell types [301]. The actual cAMP regulatory element in the *ABCA1* gene promoter has not been definitively identified, and most cases of cAMP-stimulated ABCA1 transcription have been observed in macrophages [223, 301]. It is therefore suggested that the effect of cAMP on ABCA1 expression may be specific to cell type.

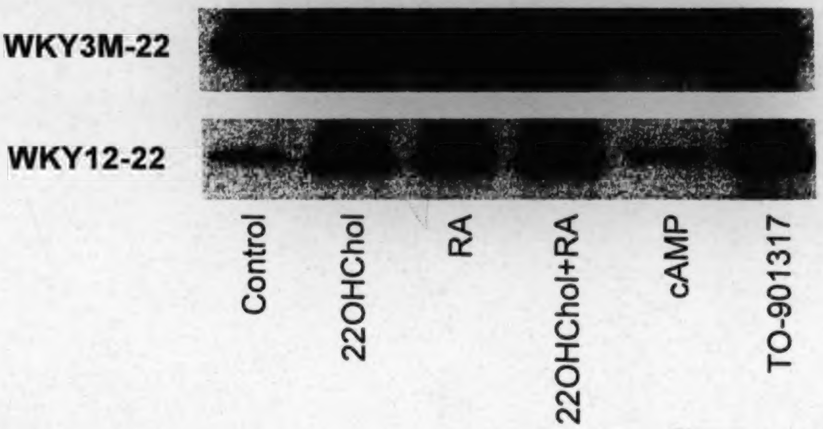


Figure 5-1. Normal regulation of ABCA1 gene expression in WKY SMC by exogenous LXR/RXR ligands. Confluent cells were incubated for 24 h in the presence of 10 μM 22(R)-hydroxycholesterol (22OHChol), 10 μM 9-cis-retinoic acid (RA), 22OHChol plus RA, 0.3 mM 8-Br-cAMP (cAMP), or 10 μM LXR agonist TO-901317 prior to determination of ABCA1 protein levels. ABCA1 protein was detected by Western blotting of 50 μg of crude membrane proteins with a rabbit polyclonal antibody to ABCA1. Results are representative of two separate experiments with similar results.

5.3.2 LXR agonist TO-901317-induced expression of ABCA1 does not correct impaired apoA-I-mediated cholesterol efflux from WKY12-22 cells

In Chapter 4, a near absence of apoA-I-mediated cholesterol efflux from WKY12-22 was ascribed to impaired upregulation of ABCA1 in response to increased cell cholesterol content. The finding that *ABCA1* in WKY12-22 cells is normally regulated by exogenous LXR/RXR ligands made it possible to test whether upregulation of ABCA1 activates WKY12-22 cells to release excess cholesterol to apoA-I.

First, the ability of WKY12-22 cells to be depleted of cholesterol available for esterification by ACAT was assessed by incubating TO-901317-treated WKY12-22 cells with apoA-I. Consistent with previous findings, cholesterol-loaded WKY3M-22 cells were actively depleted of ACAT-substrate cholesterol by

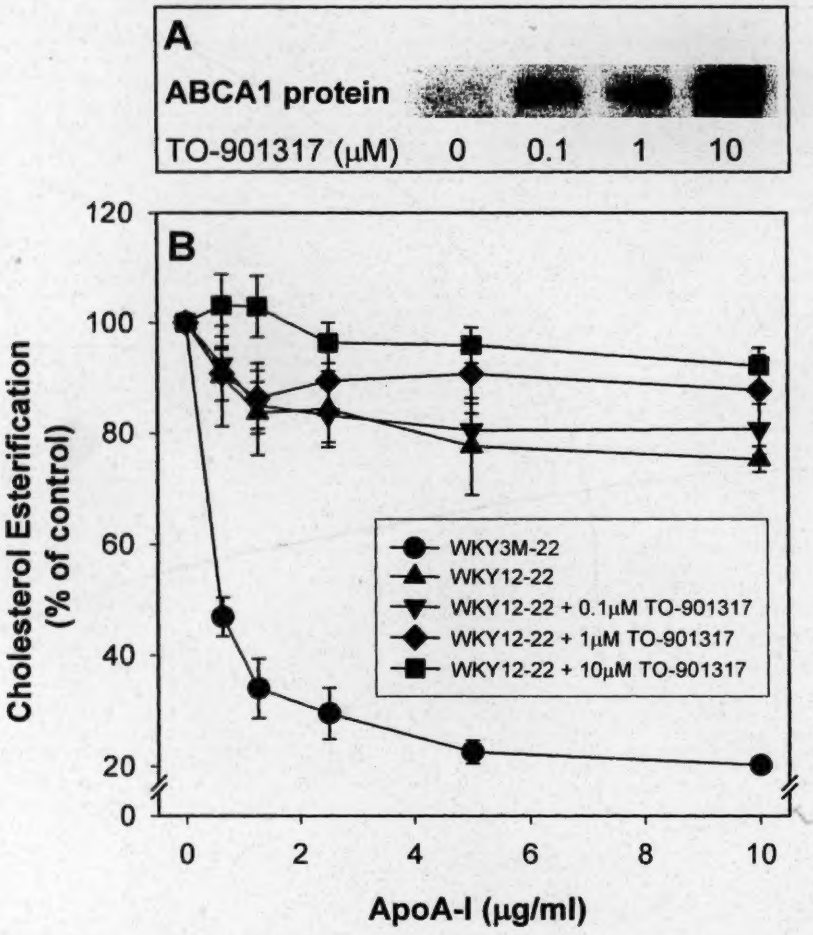


Figure 5-2. TO-901317-stimulated upregulation of ABCA1 fails to correct apoA-I-mediated depletion of cholesterol available for esterification in WKY12-22 cells. (A) Confluent cells were incubated for 24 h with the indicated concentration of LXR agonist TO-901317, and crude membrane ABCA1 protein levels were determined by Western blotting. **(B)** Confluent cells were loaded with cholesterol, equilibrated, and incubated with the indicated concentration of apoA-I for 16 h. To increase ABCA1 expression in WKY12-22 cells, the indicated concentration of TO-901317 was added during the periods of equilibration and apoA-I treatment. Cells were then incubated with [^{14}C]oleate for 1 h to measure formation of new cholesteryl [^{14}C]ester. Values are the mean ± S.D. of quadruplicate determinations expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl ester per milligram of cell protein per hour in cells treated with control medium containing no apoA-I, and representative of two separate experiments with similar results.

apoA-I, whereas cholesterol-loaded WKY12-22 cells were resistant to apoA-I-mediated depletion of ACAT-substrate cholesterol (Figure 5-2B). Cholesterol-loaded WKY12-22 cells showed increasing levels of ABCA1 protein in response to increasing concentration of TO-901317 (Figure 5-2A). Very interestingly, however, no depletion of ACAT accessible cholesterol was seen in cells treated with any concentration of TO-901317 tested, despite the increased ABCA1 protein and the known ability of ABCA1 to deplete ACAT-accessible cholesterol [88]. These results suggest either that ABCA1 in WKY12-22 SMC lacks activity, or that factor(s) other than ABCA1 necessary for lipid efflux are lacking in these cells.

Next, the effect of TO-901317 treatment on apoA-I-mediated cholesterol efflux was examined. Cholesterol-loaded WKY3M-22 cells released cholesterol to apoA-I in a apoA-I dose-dependent and saturable fashion, and the addition of 10 μM TO-901317 further enhanced the ability of WKY3M-22 cells to release cholesterol to apoA-I. In contrast, apoA-I-mediated cholesterol efflux was near absent in cholesterol-loaded WKY12-22 cells, and the addition of 10 μM TO-901317 showed no ability to increase apoA-I-mediated cholesterol efflux from these cells (Figure 5-3Aa). Treatment with 10 μM TO-901317 significantly increased both total and cell surface ABCA1 protein levels in both WKY SMC lines (Figure 5-3B), suggesting TO-901317-induced ABCA1 is targeted normally to the plasma membrane. Enhanced apoA-I-mediated cholesterol efflux from TO-901317-treated WKY3M-22 cells correlated well with the increased cell surface ABCA1 levels. However, despite a significant increase in cell surface ABCA1 in TO-901317-treated WKY12-22 cells, there was no significant increase in cholesterol efflux to apoA-I. The near absence of cholesterol efflux from WKY12-22 cells to medium was reflected in no significant changes in cellular CE and unesterified cholesterol (UC) levels (Figure 5-3Ab and Ac). The apoA-I-mediated cholesterol efflux results were consistent with the results of apoA-I-mediated depletion of ACAT-accessible cholesterol. These results together suggest that upregulation of ABCA1 is not sufficient to correct the impaired apoA-I-mediated

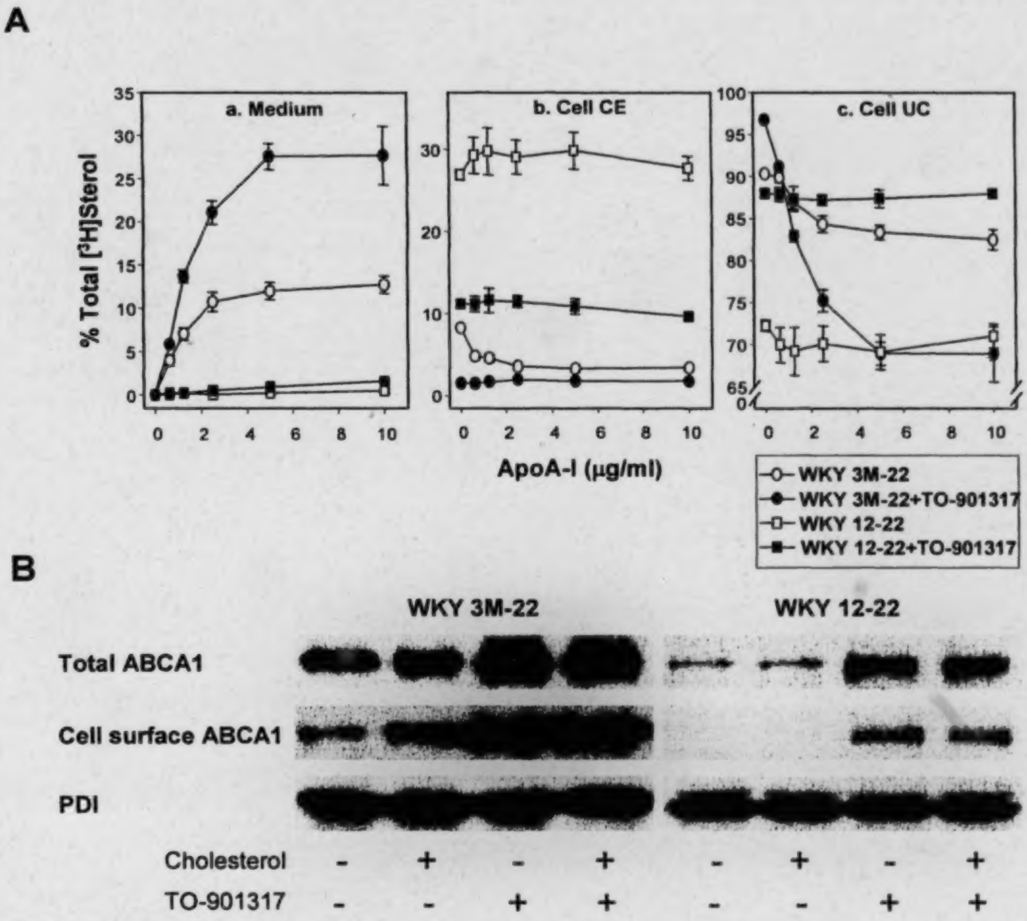


Figure 5-3. TO-901317-stimulated upregulation of ABCA1 fails to correct apoA-I-mediated cholesterol efflux in WKY12-22 cells. (A) Confluent cells labeled with [^{3}H]cholesterol during growth were loaded with cholesterol for 24 h. The cells were incubated in the absence or presence of 10 µM TO-901317 during equilibration for 24 h and incubation with the indicated concentration of apoA-I for 24 h. At the end of the incubation, the media were collected and cell cholesteryl ester (CE) and unesterified cholesterol (UC) in cells and media were analyzed for [^{3}H]sterol. Results are expressed as percentage of total cell plus medium [^{3}H]sterol in the medium (Aa), cell CE (Ab), and cell UC (Ac) following subtraction of efflux to medium containing 1 mg/ml BSA alone. Values are the mean ± S.D. of quadruplicate determinations, and representative of two separate experiments with similar results. **(B)** Cells treated equivalent to the cholesterol efflux studies were biotinylated, and total and cell surface ABCA1 levels were determined by Western blotting. Protein disulfide isomerase (PDI) levels were determined by Western blotting as a loading control. Results are representative of two separate experiments with similar results.

cholesterol efflux from WKY12-22 cells, despite apparent normal localization of the increased ABCA1 in agonist-treated cells. The absence of an effect of TO-901317 on apoA-I-mediated cholesterol efflux from WKY12-22 cells also suggests that additional LXR agonist-nonresponsive factors are necessary to facilitate apoA-I-mediated cholesterol efflux, and are lacking in WKY12-22 cells.

An interesting additional finding was that treatment with 10 μM TO-901317 markedly decreased cellular CE label and increased cellular UC label at time zero in both cell lines (Figure 5-3Ab and Ac). A similar effect of TO-901317 on the shift of cell cholesterol has also been observed in human macrophages [302]. The increased UC label in TO-901317-treated WKY3M-22 cells was removed by apoA-I (Figure 5-3Ac), indicating that TO-901317 stimulation causes a shift of cell cholesterol from the ACAT substrate pool to an apoA-I accessible pool. However, the apoA-I accessible cholesterol pool in WKY12-22 cells was not removed by apoA-I despite the presence of a significantly increased amount of ABCA1 in the plasma membrane (Figure 5-3Ac and 5-3B), providing further evidence for a missing additional factor(s) necessary for apoA-I-mediated cholesterol efflux in WKY12-22 cells. At this time, it is not clear whether the increased ABCA1 itself induces the shift of cell cholesterol, or whether other TO-901317-responsive cellular factors are responsible for this.

5.3.3 Transfection of ABCA1 fails to correct impaired apoA-I-mediated cholesterol efflux from WKY12-22 cells

The synthetic LXR agonist TO-901317 can turn on not only *ABCA1* but also many other genes containing an LXR response element in their promoter regions. This makes it difficult to make conclusions about the specific effects of the agonist on ABCA1 regulation. To examine the specific effect of ABCA1, apoA-I-mediated cholesterol efflux studies were performed using cells transfected with full-length murine ABCA1 cDNA. WKY3M-22 cells transfected with the ABCA1 construct showed an increase in ABCA1 protein and a significantly enhanced ability to release cholesterol to apoA-I, even in the absence of cholesterol loading, when compared to empty vector-transfected

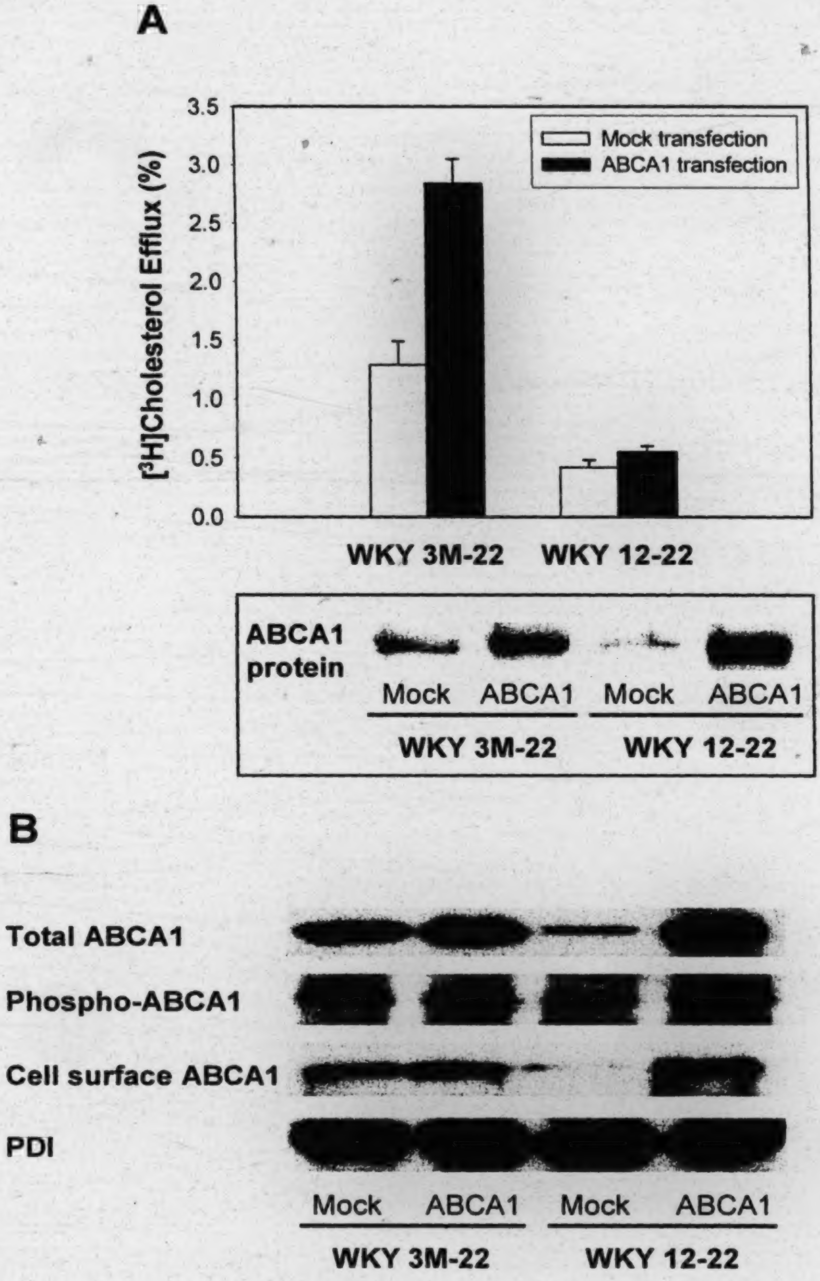


Figure 5-4. Transfection of ABCA1 fails to correct impaired apoA-I-mediated cholesterol efflux from WKY12-22 cells. (A) Cells labeled with [^{3}H]cholesterol during growth were transfected with ABCA1 construct or empty vector for 24 h, and incubated with 10 μg/ml apoA-I for 4 h. At the end of the

incubation period, the media and cells were collected and the cells were homogenized. The media and aliquots of cell homogenates were analyzed for [^{3}H]cholesterol, and 100 µg of cell proteins were used to determine total ABCA1 protein by Western blotting. Cholesterol efflux data are expressed as percentage of total cell plus medium [^{3}H]sterol in the medium and values are the mean ± S.D. of quadruplicate determinations. Results are representative of two separate experiments with similar results. **(B)** Cells grown to ~70% confluence were transfected with ABCA1 construct or empty vector for 24 h. The cells were biotinylated and lysed in RIPA buffer. One hundred micrograms of total cell protein were subjected to Western analyses for total ABCA1, and the blots were reprobed with anti-PDI antibody to verify equal loading. The levels of PKA-dependent phosphorylated ABCA1 (phospho-ABCA1) were determined by separate Western analyses with 100 µg of total cell protein using anti-phospho-(Ser/Thr) PKA substrate antibody. To determine cell surface ABCA1 levels, biotinylated proteins were purified from 2 mg of total cell protein, and 50 µl of purified samples were subjected to Western analyses for cell surface ABCA1.

control cells (Figure 5-4A). In sharp contrast, WKY12-22 cells showed no significant increase in apoA-I-mediated cholesterol efflux despite a marked increase in total cell ABCA1 protein (Figure 5-4A).

In parallel, cell surface localization and PKA-mediated phosphorylation of transfected ABCA1 were investigated to attempt to determine whether the transfected ABCA1 was processed in the same way as endogenous ABCA1. PKA-mediated phosphorylation of ABCA1 has been reported to occur constitutively and is essential for optimum lipid-transport activity of ABCA1 [159, 160]. WKY3M-22 and WKY12-22 cells transfected with ABCA1 increased both cell surface and phosphorylated ABCA1 compared to mock-transfected control cells, and WKY12-22 cells showed a higher fold increase in these parameters than WKY3M-22 cells (Figure 5-4B). The increase of total ABCA1 protein in transfected WKY12-22 cells was also confirmed by immunofluorescence detection of ABCA1 (data not shown). The absence of a significant effect of apparently normally processed ABCA1 in WKY12-22 cells on apoA-I-mediated cholesterol efflux strongly suggests that other factor(s), in addition to ABCA1, limit the ability of WKY12-22 SMC to interact with apoA-I for ABCA1-dependent cholesterol mobilization and HDL particle formation.

5.3.4 Hydrolysis of cholesteryl esters does not limit apoA-I-mediated cholesterol efflux from WKY12-22 cells

CE droplets in the cytoplasm undergo a continual cycle of hydrolysis and re-esterification as part of normal cholesterol homeostasis, and UC generated by the hydrolysis of CE is excreted from cells when extracellular cholesterol acceptors such as apoA-I or HDL are present [303-305]. To see whether the impaired apoA-I-mediated cholesterol efflux from WKY12-22 cells could be explained by a defect in hydrolysis of CE stores, I assessed hydrolysis of cholesteryl [^{14}C]oleate in the presence of an ACAT inhibitor, Sandoz 58-035, to prevent re-esterification of UC. Consistent with previous findings, cholesterol-loaded WKY12-22 cells accumulated much more cholesteryl [^{14}C]oleate compared to cholesterol-loaded WKY3M-22 cells (Figure 5-5A). Both WKY3M-22 and WKY12-22 cells showed a time-dependent decrease in the level of cholesteryl [^{14}C]oleate in the presence of Sandoz 58-035 (Figure 5-5A). When I expressed the hydrolysis rates of cholesteryl [^{14}C]oleate as percentage of 0 h incubation, WKY12-22 cells showed a somewhat slower rate of hydrolysis at early time points compared to WKY3M-22 cells, but there was no difference between the two cell lines after 48 h incubation (Figure 5-5B). It is also notable that the absolute amount of CE hydrolyzed at all time points is much more in WKY12-22 cells than in WKY3M-22 cells. The presence of 10 µg/ml apoA-I in the culture medium did not affect the rate of hydrolysis of cholesteryl [^{14}C]oleate (Figure 5-5A and B). This was previously shown to be the case with HDL as the lipid acceptor by Brown *et al.* [303]. The abundance of CE hydrolyzed in WKY12-22 cells and the lack of dependence on apoA-I suggest that cholesterol efflux is not limited by hydrolysis of accumulated CE in these cells.

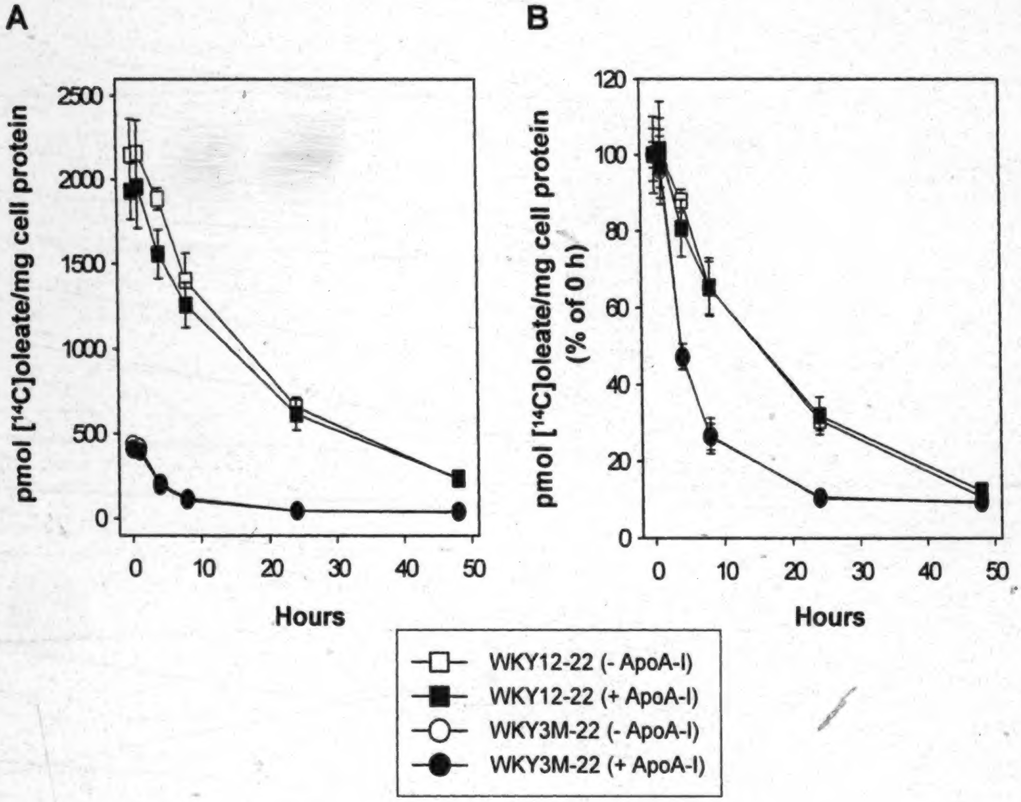


Figure 5-5. Hydrolysis of cholesteryl esters in WKY SMC in the presence of ACAT inhibitor. Confluent cells were labeled with [^{14}C]oleate during cholesterol loading and equilibration. The cells were incubated up to 48 h in the presence of 2 µg/ml ACAT inhibitor Sandoz 58-035 with or without 10 µg/ml apoA-I. After the indicated intervals, cellular lipids were extracted and analyzed for cholesteryl [^{14}C]oleate. Values are the mean ± S.D. of quadruplicate determinations expressed as picomoles of [^{14}C]oleate incorporated into cholesteryl ester per milligram of cell protein **(A)** or as percentage of 0 h incubation **(B)**. Open symbols overlapped with filled symbols are not shown. Results are representative of two separate experiments with similar results.

5.3.5 Factors in addition to ABCA1 predict apoA-I binding to cells

It has been suggested that apoA-I binds directly to ABCA1 and/or associates with specific lipid domains created by the action of ABCA1 in the plasma membrane to mediate lipid efflux [116, 121, 125, 224, 306]. However, in Chapter 4 I observed that WKY12-22 cells loaded with cholesterol showed significantly increased binding of apoA-I despite no significant increase in ABCA1 expression. This suggests that ABCA1 may be not the determining factor for the apoA-I-cell binding interaction. To examine more directly the effect of ABCA1 expression level on apoA-I binding, cells were transfected with ABCA1 construct and their ability to bind apoA-I was assessed. Mock-transfected WKY12-22 cells had markedly lower binding of apoA-I compared to similarly-treated WKY3M-22 cells (Figure 5-6), which might be attributed to the lower expression of ABCA1 in WKY12-22 cells. A significant increase in ABCA1 protein in both cell lines following transfection, however, resulted in no significant increase in apoA-I binding in either cell line. These results, combined with the cell surface biotinylation and PKA-dependent ABCA1 phosphorylation results, strongly suggest that factors in addition to ABCA1 and lacking in WKY12-22 intimal-phenotype cells predict binding of apoA-I to cells.

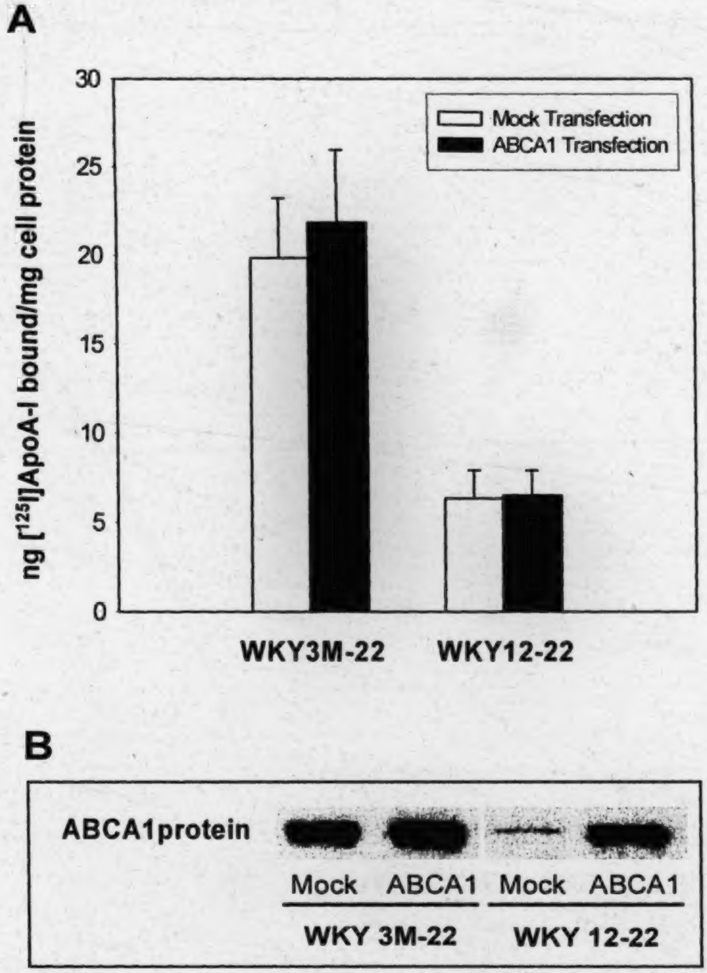


Figure 5-6. Transfection of ABCA1 fails to increase apoA-I bindings in WKY SMC. Cells transfected with ABCA1 construct or empty vector for 24 h were incubated for 2 h at 4 °C with 10 µg/ml ^{125}I-apoA-I ± 200 µg/ml unlabeled apoA-I. The cells were rinsed extensively and assessed for radioactivity associated with cells **(A)**. Cell surface ^{125}I-apoA-I bound per mg of cell protein was determined by subtracting values in the presence of the unlabeled apoA-I and values are the mean ± S.D. of quadruplicate determinations. To see the efficiency of transfection, cells transfected for 24 h were harvested and the expression levels of crude membrane ABCA1 were determined by Western blotting **(B)**. Results are representative of three separate experiments with similar results.

5.4 Discussion

The predominance of epithelioid-shaped synthetic SMC in the atherosclerotic intima has motivated intensive research to understand what properties of these cells are linked to atherogenesis. Numerous studies have attempted to determine cellular factors leading intimal SMC to be atherogenic by comparing these cells to spindle-shaped contractile SMC, the major SMC subtype in the normal artery medial layer (reviewed in [190]). Our previous findings that epithelioid SMC showed impaired upregulation of ABCA1 in response to increasing cellular cholesterol content, and therefore impaired apoA-I-mediated lipid efflux, suggested impaired ABCA1 regulation or expression alone might explain the overaccumulation of cholesterol in these cells. In the current studies, we show that ABCA1 is regulated normally in epithelioid WKY12-22 SMC by exogenous LXR and RXR ligands, but that increased ABCA1 protein levels seen in LXR agonist-treated or transfected cells fails to correct the impaired apoA-I-mediated cholesterol efflux from these cells. Low binding capacity of WKY12-22 cells to apoA-I and the absence of a significant effect of transfected ABCA1 on apoA-I-cell association (Figure 5-6) suggests that in addition to ABCA1, an additional cellular factor or factors facilitating the apoA-I-cell association are lacking in WKY12-22 cells.

It has been believed that oxysterols produced in cholesterol-loaded cells are the key physiological regulators turning on LXR target genes, including ABCA1, to activate the cholesterol efflux pathway [134]. Despite the accumulation of CE, and the presence of a normally responsive ABCA1 gene in WKY12-22 cells (Figure 5-1), ABCA1 expression was not upregulated in response to cholesterol loading. This suggests that WKY12-22 epithelioid cells may be defective in the production of oxysterols. In support of this speculation, Shanahan *et al.* [307] reported that de-differentiated human aortic SMC expressed a very low level of 27-hydroxylase, which catalyzes the rate-limiting step in the production of 27-hydroxycholesterol, the most abundant oxysterol in atherosclerotic lesions.

The failure of increased ABCA1 protein levels following treatment with an LXR agonist or transfection with ABCA1 construct to correct the impaired apoA-I-mediated cholesterol efflux from WKY12-22 cells strongly suggests that ABCA1 alone or other LXR-target genes are not sufficient to release cholesterol from WKY12-22 cells to apoA-I. Since hydrolysis of CE was normal in WKY12-22 cells (Figure 5-5), it is plausible to speculate that the transport of UC to the plasma membrane might be not normal in WKY12-22 cells. In support of this idea, TO-901317-treated WKY12-22 cells accumulated UC generated by hydrolysis of CE without releasing it to apoA-I, suggesting cellular UC may not be mobilized to apoA-I-accessible sites in the plasma membrane. Kellner-Weibel *et al.* [305] reported that UC generated by hydrolysis of CE is transported through acidic vesicles to the plasma membrane. Therefore, the level of plasma membrane cholesterol and the presence of normal acidic vesicle transport system in WKY12-22 cells remain to be investigated. If WKY12-22 cells are impaired in the production of oxysterols, it would also be interesting to explore whether the impaired production of oxysterols is also due to an abnormal transport system of the acidic vesicles.

Although the low binding capacity of WKY12-22 cells to apoA-I is consistent with impaired cholesterol efflux from WKY12-22 cells, apoA-I binding did not correlate with ABCA1 protein levels. Cholesterol-loaded WKY3M-22 cells showed increased ABCA1 expression and apoA-I binding capacity, but cholesterol-loaded WKY12-22 cells significantly increased apoA-I binding capacity without a significant increase in ABCA1 expression (Figure 4-2 and 4-9). Transfection of ABCA1 into both cells lines without cholesterol loading increased ABCA1 protein levels, but the binding capacity to apoA-I was not increased in either cell line (Figure 5-6). These results suggest that ABCA1 alone is not sufficient to facilitate apoA-I-cell binding, and that cholesterol-stimulated modulation of cells, including changes in lipid composition in the plasma membrane and activation/induction of cellular factors in addition to ABCA1, are involved in facilitating apoA-I-cell binding. Lipid composition in the plasma membrane is influenced by the traffic of cytosolic UC to the plasma membrane, again suggesting it will be important to examine whether WKY12-22 cells have a normal transport system for UC-containing acidic vesicles.

The presence of additional apoA-I binding factors has been suggested previously. Fitzgerald *et al.* [308] reported that mutation in the highly conserved C-terminal VFVNFA motif of ABCA1 abolishes its lipid efflux activity and cross-linking ability to apoA-I without affecting its localization to the plasma membrane. The authors speculated that the VFVNFA motif may be a protein-protein interaction domain that recruits additional factors that promote the apoA-I-ABCA1 interaction and lipid efflux to apoA-I. Burgess *et al.* [309] speculated that trypsin-sensitive extracellular matrix components of THP-1 and J777 macrophages facilitate binding of these cells to apoA-I in promoting cholesterol efflux. Considering the importance of the apoA-I-cell interaction in initiating and promoting ABCA1-dependent lipid efflux, it is crucial to identify this additional factor or factors involved in apoA-I-cell binding. The marked difference in apoA-I-binding capacity between WKY3M-22 and WKY12-22 cells provides an excellent model to explore additional factors required for the apoA-I-cell interaction.

Several protein kinases have been reported to influence the function and stability of ABCA1. We showed that PKA-mediated phosphorylation of ABCA1, constitutive and essential for optimum lipid-transport activity of ABCA1 [159], was present in transfected ABCA1 in both WKY12-22 and WKY3M-22 cells (Figure 5-4B). We therefore concluded that impaired PKA-dependent phosphorylation of ABCA1 was unlikely to explain the absence of increased cholesterol efflux in ABCA1-transfected WKY12-22 SMC. In addition, total and cell surface ABCA1 were similar in ABCA1 transfected WKY12-22 and WKY3M-22 SMC (Figure 5-4B), suggesting inappropriate trafficking/localization of ABCA1 in transfected WKY12-22 SMC also did not explain the failure to correct apoA-I-mediated cholesterol efflux in these cells.

Tang *et al.* [160] reported that the apoA-I-cell interaction triggers the autophosphorylation of Janus Kinase 2 (JAK2), and that phosphorylated JAK2 in turn phosphorylates another protein to stimulate ABCA1-dependent lipid efflux. The poor apoA-I-binding capacity of WKY12-22 cells, however, suggests that

WKY12-22 cells would be defective in activating JAK2 and therefore in JAK2-mediated stimulation of ABCA1 activity. The authors reported that ABCA1 is not the direct target of the activated JAK2, and suggested that another protein phosphorylated by JAK2 would stimulate ABCA1 functions. The marked difference in apoA-I-binding capacity between WKY12-22 and WKY3M-22 cells would also be a good model to search which protein is targeted by phosphorylated JAK2.

Several synthetic LXR agonists have been developed and their effects on cholesterol efflux, especially increased ABCA1 expression and activity, have been well studied in cell and mouse models (reviewed in [34, 134]). These promising results have given impetus to the development of LXR activators for the treatment of atherosclerosis. Our results suggest, however, that an LXR-agonist induced increase in epithelioid intimal SMC ABCA1 expression would fail to correct impaired apoA-I-mediated lipid efflux from these cells. This finding highlights the urgency of identifying the additional cellular factor or factors required for a productive ABCA1-apoA-I interaction, to effectively design novel therapies to decrease the burden of excess cholesterol in atherosclerotic SMC.

5.5 Conclusions

The results presented here suggest that WKY12-22 cells used as a model of atherogenic intima epithelioid SMC may be defective in the production of oxysterols, resulting in impaired ABCA1 regulation and apoA-I-mediated cholesterol efflux. More importantly, the data also suggest that in addition to ABCA1, a factor or factors involved in apoA-I-cell binding and necessary for the apoA-I-ABCA1 interaction are lacking in WKY12-22 cells. The marked difference in response to apoA-I between WKY3M-22 and WKY12-22 cells suggests a mechanism for accumulation of cholesterol in atherosclerotic lesion SMC, and provides an excellent model to study the additional cellular requirements for a productive apoA-I-cell interaction to form HDL particles.

CHAPTER 6:

Conclusions and Future Directions

6.1 Summary

6.1.1 ATP-binding cassette transporter A1 (ABCA1) deficiency in Niemann-Pick type C (NPC) disease

Cellular cholesterol homeostasis is stringently controlled through the regulation of genes and proteins involved in the biosynthesis, influx, efflux, and esterification of cholesterol. The intracellular cholesterol trafficking defect in NPC disease, a neurovisceral disorder characterized by the accumulation of unesterified cholesterol and glycosphingolipids in late endosomes/lysosomes, results in impaired regulation of biosynthesis, influx, and esterification of cholesterol. ABCA1 mediates the efflux of cholesterol and phospholipids to lipid-poor HDL apolipoproteins, in particular apolipoprotein A-I (apoA-I), in the process of high density lipoprotein (HDL) formation, and is also regulated by cellular cholesterol content. The alterations in cholesterol homeostatic mechanisms in NPC disease led us to hypothesize that ABCA1 regulation and HDL formation would also be impaired in this disease.

In Chapter 3, the effect of NPC1 deficiency on the expression and function of ABCA1 were studied to test the hypothesis. The function of ABCA1 on lipid efflux was determined by examining the ability of apoA-I to remove pools of radiolabeled cellular cholesterol and phospholipids in human fibroblasts derived from $NPC1^{+/+}$, $NPC1^{+/-}$, and $NPC1^{-/-}$ subjects. Efflux of radiolabeled low density lipoprotein (LDL)-derived, non-lipoprotein-derived, plasma membrane and newly synthesized pools of cell cholesterol by apoA-I were all diminished from $NPC1^{-/-}$ cells, as was efflux of phosphatidylcholine (PC) and sphingomyelin (SM). $NPC1^{+/-}$ cells showed intermediate levels of lipid efflux compared to $NPC1^{+/+}$ and $NPC1^{-/-}$ cells. Consistent with the ABCA1-dependent lipid efflux results, basal and cholesterol-stimulated ABCA1 mRNA and protein levels were markedly decreased in $NPC1^{-/-}$ cells compared to $NPC1^{+/+}$ and $NPC1^{+/-}$ cells. Correlated with impaired ABCA1-dependent lipid efflux from $NPC1^{-/-}$ cells to apoA-I for HDL particle formation, we observed for the first time that plasma HDL-cholesterol levels in 17 of 21 (81%) $NPC1^{-/-}$ subjects was below the currently identified lower limit of normal level (1.03 mmol/liter). These results indicate that the cholesterol trafficking defect in NPC disease impairs ABCA1 regulation and therefore ABCA1-dependent lipid efflux, which is responsible for the low HDL-cholesterol in the majority of NPC subjects, and at least partially responsible for the accumulation of cellular lipids in this disorder.

To examine whether ABCA1 expression levels in NPC disease correlate with binding of apoA-I to cells, cholesterol-loaded and non-cholesterol-loaded cells were incubated with ^{125}I-labeled apoA-I. *NPC1*$^{+/-}$ cells showed the highest binding capacity to apoA-I, with *NPC1*$^{+/+}$ and *NPC1*$^{-/-}$ cells showing a similar binding capacity despite markedly different levels of ABCA1 expression. These results suggest that the association of apoA-I with cells is facilitated by factors other than or in addition to ABCA1.

Subsequent work done in our laboratory, in which I was indirectly involved, showed that treatment of *NPC1*$^{-/-}$ cells with a synthetic liver X receptor (LXR) agonist, TO-901317, corrects ABCA1 expression as well as PC and LDL-derived cholesterol efflux to apoA-I. Consistent with this, we observed decreased formation of large HDL particles by *NPC1*$^{-/-}$ cells as assessed by 2-dimemsional gel electrophoresis, with correction to a normal pattern of HDL species following stimulation with TO-901317. These results suggest that the *NPC1* mutation-induced impairment of ABCA1 regulation and HDL particle formation can be corrected by exogenous LXR agonists, which provides a potential therapeutic mechanism for correction of lipid accumulation and trafficking in NPC disease.

6.1.2 ABCA1 deficiency in atherosclerotic smooth muscle cells (SMC)

A biochemical marker of atherosclerotic lesions is the accumulation of cholesteryl esters (CE) in the innermost layer of the artery, the intima, and several cell types including macrophages, endothelial cells, and SMC contribute to the enlargement of this normally acellular layer. The initial recruitment of SMC to the intima could be a defensive response to arterial injury, because fibrous intimal tissue produced primarily by intimal SMC is thought to enclose injurious agents and assist in resolution of the injury. However, intimal SMC, like intimal macrophages, take up modified cholesterol-rich lipoproteins such as oxidized

LDL and become CE-enriched SMC foam cells [183, 185]. Heterogeneity of arterial SMC in morphology and biochemical properties has been identified in various species including human. Spindle-shaped contractile SMC comprise the major SMC subtype in the normal medial layer of arteries, whereas epithelioid-shaped synthetic SMC are the mayor subtype of intimal SMC. The accumulation of CE in the intima but not in the media led us to hypothesize that intimal-phenotype epithelioid SMC are impaired in ABCA1-dependent cholesterol efflux.

In Chapter 4, the ability of epithelioid and spindle SMC lines to bind and release lipids to apoA-I was examined to test the hypothesis. Spindle SMC showed active efflux of radiolabeled non-lipoprotein-derived cholesterol, PC, and SM to apoA-I, and were actively depleted of cholesterol available for esterification by acyl-CoA:cholesterol acyltransferase (ACAT) by apoA-I. Remarkably, however, epithelioid SMC showed a near absence of these ABCA1-dependent lipid efflux activities despite accumulation of excess CE. The impaired radiolabeled cholesterol efflux to apoA-I from epithelioid SMC was confirmed by measuring cholesterol mass in medium and cells. Consistent with the lipid efflux results, spindle SMC showed a relatively high (compared to skin fibroblasts) basal level of ABCA1 mRNA and protein, and increase in ABCA1 expression with cholesterol loading, whereas epithelioid SMC showed a very low basal level of ABCA1 mRNA and protein and no significant increase in response to cholesterol loading. Binding of apoA-I to both cell lines was significantly increased with cholesterol loading, however epithelioid SMC showed a markedly low apoA-I-binding capacity under both basal and cholesterol-loaded conditions compared to spindle SMC. These results suggest intimal-phenotype epithelioid SMC are impaired in upregulation of ABCA1 in response to increasing cell cholesterol content, and therefore in ABCA1-dependent lipid efflux, whereas spindle SMC actively regulate ABCA1-dependent lipid efflux to maintain cellular cholesterol homeostasis.

In Chapter 5, ABCA1 expression in epithelioid SMC was upregulated by treating cells with TO-901317 or transfecting cells with full-length murine ABCA1 cDNA to examine whether increased ABCA1 can correct the impaired lipid efflux.

TO-901317-stimulated epithelioid SMC significantly increased total and cell surface ABCA1 protein compared with non-stimulated control cells, however the impaired depletion of cellular cholesterol available for esterification by apoA-I and impaired cholesterol efflux to apoA-I were not corrected. Epithelioid SMC transfected with ABCA1 construct showed a marked increase in total ABCA1 normally phosphorylated by PKA, and in cell surface ABCA1, compared with mock transfected control cells, however the impaired ABCA1-dependent cholesterol efflux to apoA-I was again not corrected. These results strongly suggest that in addition to ABCA1, other factor or factors involved in ABCA1-dependent lipid efflux to apoA-I are lacking in intimal-phenotype epithelioid SMC but present in spindle SMC. The normal hydrolysis of CE accumulated in epithelioid SMC in the presence of ACAT inhibitor excluded the possibility of defective CE hydrolase activity as a cause of impaired cholesterol mobilization from these cells. Following transfection with ABCA1 construct, increased cell surface ABCA1 resulted in no significant increase in apoA-I binding to either epithelioid or spindle SMC, but spindle SMC showed a persistent higher binding capacity. These results further suggest that a factor or factors facilitating the interaction between cells and apoA-I is lacking in epithelioid SMC.

Impaired ABCA1-dependent lipid efflux from intimal-phenotype epithelioid SMC suggests a mechanism for the accumulation of excess cholesterol in these cells in the intima of atherosclerotic lesions. Our finding that increased ABCA1 in intimal-phenotype epithelioid SMC is not sufficient to correct impaired apoA-I-mediated lipid efflux from these cells indicates how critical it is to identify the additional cellular factor or factors necessary for a productive apoA-I-cell interaction to decrease cholesterol accumulation in atherosclerotic lesions. The marked difference in ABCA1-dependent lipid efflux between spindle SMC and epithelioid SMC provides an excellent model to identify these additional requirements for a productive apoA-I-cell interaction leading to HDL formation.

6.2 Future directions

An exogenous LXR ligand, TO-901317, corrected impaired regulation and function of ABCA1 in *NPC1*$^{-/-}$ fibroblasts, however increased ABCA1 in intimal-phenotype epithelioid SMC induced by TO-901317 or transfection was not active in releasing overloaded CE to apoA-I despite appropriate localization and phosphorylation of the increased ABCA1 protein. These results suggest the presence of additional factors to activate ABCA1-dependent cholesterol efflux to apoA-I, and are absent in epithelioid SMC. ApoA-I binding assays showed that the ability of human fibroblasts (Figure 3-6) and rat SMC (Figure 5-6) studied in this thesis to bind apoA-I was not determined by cellular ABCA1 protein levels, and that epithelioid SMC had markedly low apoA-I binding capacity compared to spindle SMC. If the initial interaction between cells and apoA-I is defective, subsequent cellular ABCA1 actions are ineffective, which seems to be the most likely explanation for the near absence of lipid efflux from epithelioid SMC following upregulation of ABCA1 by LXR agonist or transfection. These results suggest that additional factors in ABCA1-dependent lipid efflux facilitate the initial interaction between apoA-I and cells.

This speculation proposes the most important future study, to examine what additional factors facilitate the apoA-I-cell interaction and that might be present in spindle but absent or diminished in epithelioid SMC. Possible apoA-I interacting factors include cell surface lipids, cell surface proteins, and extracellular matrix proteins. Methods for isolation of plasma membranes [310] and extracellular matrix proteins [311-313] have been established. Analysis of proteins using two-dimensional gel electrophoresis and analysis of lipid composition using gas chromatography and/or high-performance lipid chromatography could provide useful information to narrow down target molecules. The presence of specific proteins interacting with apoA-I can be tested by protein cross-linking and immunoprecipitaion of apoA-I. To increase the sensitivity of apoA-I detection, ^{125}I-labeled apoA-I can be used in protein cross-linking studies. Tang *et al.* [160] reported that the apoA-I-cell interaction stimulates autophosphorylation of Janus kinase 2, which in turn phosphorylates proteins other than ABCA1 to enhance apoA-I-mediated lipid efflux. This study provides another tool to search cellular factors necessary for ABCA1 actions. Janus kinase 2-stimulated phosphorylation would be expected to occur normally in spindle SMC but possibly not or less so in epithelioid SMC, because apoA-I binding by epithelioid SMC was much lower than spindle SMC.

Although a minimal amount of cholesterol and phospholipid efflux from epithelioid SMC to apoA-I suggests apoA-I fails to form even small sized HDL particles from epithelioid SMC, analysis of HDL particle species in cell-conditioned media by two-dimensional non-denaturing gel electrophoresis might provide further insight into the apoA-I-cell interaction.

Epithelioid SMC showed not only impaired apoA-I-mediated cholesterol efflux but also very low apoA-I-independent passive diffusion of cholesterol to the medium compared to spindle SMC (Figure 4-4). Passive diffusion occurs down a concentration gradient of unesterified cholesterol (UC) from the cell surface to acceptors in the medium, suggesting epithelioid SMC may contain less cell surface UC than spindle SMC. This speculation can be tested by assessing levels of cell surface UC by treating cells with cholesterol oxidase and assaying for cellular cholestenone [123, 305].

Treatment with TO-901317 increased cellular UC content and ABCA1 expression in WKY SMC (Figure 5-3). The increased UC in spindle SMC was removed by apoA-I, but epithelioid SMC failed to release UC to apoA-I (Figure 5-3). Most cellular UC is normally present in the plasma membrane, where it can be released to apoA-I via the actions of ABCA1. The absence of apoA-I-mediated cholesterol efflux from TO-901317-treated epithelioid SMC suggests a potential defect in intracellular trafficking of UC to the plasma membrane in these cells. This possibility could be tested by studying intracellular trafficking of a fluorescent cholesterol analog. The movement of fluorescent cholesterol over time under a fluorescence microscope would provide insight into intracellular cholesterol trafficking as well as assessment of cell surface UC. ABCA1 is involved in intracellular cholesterol distribution [123], therefore it would be interesting to see how transfected ABCA1 in epithelioid SMC influences cholesterol distribution. It will be also necessary to examine the role of other proteins involved in the traffic of cholesterol to the plasma membrane, such as caveolins [314, 315].

ABCA1 is upregulated in response to increasing cell cholesterol content, but the actual molecules turning on *ABCA1* transcription are oxysterols. *NPC1*$^{-/-}$ fibroblasts were reported to be defective in production of oxysterols [231], and our data suggested epithelioid SMC are also defective in production of oxysterols. Cellular oxysterol levels can be measured by gas chromatography connected to mass spectrometry [231]. Alternatively, the expression levels of key enzymes in the synthesis of oxysterols can be measured, such as 27-hydroxylase and 25-hydroxylase [150].

Our cell studies demonstrate that impaired regulation and function of ABCA1 contribute to accumulation of CE in intimal-phenotype epithelioid SMC. Further studies are required to identify whether ABCA1 expression is also low in human atherosclerotic intima SMC compared to medial SMC. In situ hybridization analysis of ABCA1 in artery sections can be performed to determine ABCA1 mRNA levels. Immunohistochemical analysis of SMC markers, such as smooth muscle α-actin and smooth muscle myosin heavy chain, on sections adjacent to those used for in situ hybridization will differentiate ABCA1 in SMC from ABCA1 in macrophages.

6.3 Concluding remarks

The findings of this thesis describe significant advances in our understanding of NPC disease and proatherogenic SMC. Our finding of ABCA1 deficiency in both NPC disease cells and intimal-phenotype epithelioid SMC further emphasize the crucial role of ABCA1 in cholesterol homeostasis. The ability of correction of ABCA1 expression to bypass the NPC1 mutation in mobilizing cell lipids provides a potential therapeutic mechanism for correcting cholesterol accumulation in NPC disease. This work also provides strong evidence for the presence of an additional factor or factors required for ABCA1-dependent lipid efflux, which opens diverse and exciting avenues for future research.

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